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ANNUAL REPORT

FY95

94 to September 30, 1995

PART 2

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00876-05 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Determinants of Childhood Poison Ingestion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	P. Moyer	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Medicine and Dentistry, University of Rochester (N.G. Hildreth, R.A. Lawrence, G.S. Liptak)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.22	0.21	0.01

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Determinants of Childhood Poison Ingestion study investigates the relationship between social and behavioral factors and risk of childhood ingestions of household cleaning and home repair and maintenance products.

Project Description

Objectives:

The specific aims of the study are: 1) to determine the sociodemographic, predisposing, life events and situation and social network factors, parent health protective behaviors for children, and household hazards that affect childhood poison ingestion; and 2) to examine the relative contributions of these factors to poison ingestion. Funding for this project was provided by the HBL, CRMC, NICHD to the Principal Investigator at the time she was Associate Professor, Department of Pediatrics, University of Rochester. Specifically, the PRB, DESPR is responsible for all analysis and dissemination of study findings.

Methods Employed:

Using a case-control design, a proportionate systematic random sample of cases (10 months to 5 years of age; N = 326) were selected from calls for assistance to Poison Control or visits to pediatric emergency department for ingestions of household cleaning or home repair and maintenance products. Controls matched on age of child, N = 326) were obtained from community pediatric practices (N = 15). The primary caretaker responsible for the health of the child was interviewed within two weeks of the ingestion or control selection.

Adjusted analyses for separate models of major study dimensions employed multiple logistic regression. The presence of covariates yielded nonsignificant demographic effects (mother's age, education and occupational prestige, family income, and marital status; child's gender) with the exception that mothers working full-time were less likely than those not employed to have a case child (OR = .49, 95% CI = 0.32, 0.74). There were no case-control differences associated with ingestions of other substances or number of other types of injuries, where as regular daytime care in the child's own home compared to a day care center produced a significant effect (2.15, 1.02, 4.51). There was an association between reports of a greater number of major frequent life events and being a case (OR = 1.38, 95% CI = 1.11, 1.70) as well as having living fewer months in the family's current home. The presence of a salient life event was more likely to be reported by mothers of controls (OR = .64, 95% CI = 0.42, 0.98). When adjusted for measures of social network and network interaction, more interaction with a spouse/partner about the child yielded a protective effect (OR = .28, 95% CI = 0.08, 0.96) and subjects interacting more frequently with relatives other than the spouse or friends about the child were more likely to be a case than a control (OR 1.03, CI = 1.00, 1.06). A model containing measures of child risk behaviors and mother's hazard behaviors showed a significant association for a greater number of child risk behaviors and ingestion (OR = 1.58, 95% CI = 1.15, 2.17).

Significance to Biomedical Research and the Program of the Institute:

The study findings will provide recommendations for intervention strategies to reduce risk factors for ingestion which can be used in subsequent experimental studies to determine their effectiveness and contribution to the field of childhood injury prevention.

Proposed Course:

Manuscript preparation is now in progress.

Publications:

None to date.

Contract:

No contracts will be issued for this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00877-05 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prevention and Education in CHD Reduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	R. DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Medicine, Northwestern University (P. Greenland) and School of Medicine and Dentistry, University of Rochester (N.G. Hildreth, C. Cox)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.10	0.10	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Prevention and Education in Coronary Heart Disease (CHD) Reduction study investigates issues related to the future design and testing of behavioral and educational strategies to help children and adolescents reduce risk behaviors and acquire and practice positive health behaviors.

Project Description

Objectives:

The major goal of this research is to identify the unintended functional and psychological consequences of CHD risk identification. The specific aims of the study are: 1) to identify whether risk identification influences labeling effects in terms of decreased physical and role functioning and untoward health perceptions; and 2) to determine whether false reassurance occurs for individuals informed they are not at-risk as operationalized by undertaking poorer life style behaviors (e.g., cigarette smoking, unhealthy diet, sedentary habits) and reduced perceptions of health risks. Funding for this project was provided by the DHVD, NHLBI to Dr. Maiman at the time she was Associate Professor, Department of Pediatrics, University of Rochester and Co-Principal Investigator on the project. The PRB, DESPR is responsible for analysis and dissemination of study findings from the screening program related to functional, behavioral, and psychological outcomes.

Methods Employed:

7,284 individuals attended public cholesterol screening and 2,109 with elevated levels were referred for retesting and diagnosis. A 2 X 3 factorial design (health professional or lay counselor X incentive offer, reminder letter or control) was employed to examine whether interventions affect compliance with referral. In addition, 3 surveys (baseline, 5 months after screening, and one year after screening) collected information on sociodemographics, risk factors, social support and health perceptions. Cholesterol reassessments were conducted one year after screening. A comparison group of non-elevated subjects was included in the follow-up observations, yielding a data set of 3,352.

Significance to Biomedical Research and the Program of the Institute:

Evidence suggests that behaviors associated with increased CVD risks (cigarette smoking, high fat and high cholesterol diet, sedentary habits) are acquired early in life and may accelerate the development of CVD. Primary prevention programs may prevent CVD, delay the onset and reduce the severity of the disease and associated costs of medical care. However, research is needed to develop strategies to help young people reduce risk behaviors and acquire and practice positive health behaviors. Accompanying such efforts is the need to alleviate possible unintended and negative consequences of such interventions, such as labeling and false reassurance. The information from this project will contribute to the development by the PRB of a program of CVD risk reduction interventions for children and adolescents in which behavioral and educational strategies will be used.

Proposed Course:

Data have been analyzed and manuscripts are in preparation for submission for publication.

Publications:

None on major questions of interest to PRB available at this time.

Contract:

No contracts will be issued for this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2100-04 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prenatal Care Utilization and Adverse Health Behaviors During Pregnancy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research involves a program of studies employing behavioral and social support interventions to improve early entry to prenatal care and adherence to the visit schedule and decrease adverse health behaviors during pregnancy. The studies will focus on adolescent females who are at high risk of obtaining late or no prenatal care and particularly vulnerable to negative health behaviors (e.g., smoking, alcohol abuse, poor nutritional choices, illicit drug use) that are associated with poor pregnancy and birth outcomes.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The overall goal of the program of studies is to help prevent negative outcomes associated with adolescent pregnancy.

Methods Employed:

The first study in this series will focus on utilization of prenatal care. A school-based program to increase early entry to prenatal care visits is planned. The next study will build on the efficacy of this intervention to promote cessation of negative health behaviors.

Significance to Biomedical Research and the Program of the Institute:

Healthy People 2000 objectives indicate the need to improve early entry to prenatal care and reduce adverse health behaviors during pregnancy.

Proposed Course:

A concept paper is being prepared for a panel of experts and an RFC will be written.

Publications:

None.

Contract:

A contract will be developed for the project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 2101-04 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Increasing Preventive Actions for Childhood Injury

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.40	0.40	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major purpose of the Increasing Preventive Actions for Childhood Injury study is to develop and test an intervention to reduce parental unsafe practices for childhood injuries in the home. A multicomponent preventive intervention for pediatric health care providers and parents will be developed and evaluated.

Project Description

Objectives:

The primary analysis of interest will be to assess the effect of the interventions on level of unsafe practices in home by parents related to childhood injuries. Additional major areas of interest are to determine the effect of the provider intervention on physician advocacy and competency in injury prevention and on parent participation in the follow-up injury prevention program.

Methods Employed:

An experimental design will be employed with physicians assigned to no treatment (control) or injury prevention education intervention. A parent-child sample will be obtained for each pediatrician and randomized to either follow-up injury prevention program or standard treatment. The study will focus on children 10 months to 5 years of age.

Significance to Biomedical Research and the Program of the Institute:

Research on educational and motivational strategies for injury control needs to respond to complex issues including the mechanism of effective approaches and identification of subpopulations who may respond differential to such intervention. Studies have demonstrated the influential roles of physician information, attitudes and behavior on the adoption of recommended practices of patients. It appears appropriate to enhance to the capacity of physicians to take an active role as motivators and advocates for injury prevention and to recognize the social and behavioral factors associated with high risk families.

Proposed Course:

A concept review has been completed and approved by an independent panel of reviewers. The RFC is being written for internal NICHD review and approval.

Publications:

None yet.

Contract:

A contract will be developed for the project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 2107-04 PRB

PERIOD COVERED

October 1, 1994 through August 1, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Children and Adolescent Trial of Cardiovascular Health (CATCH)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

University of Texas at Houston (G.S. Parcel); University of Minnesota (C. Perry); University of California at San Diego (P. Nader); Tulane University (L. Weber)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The study is a test of the effectiveness of a multi-component, school-based intervention to modify CVD risk factors among preadolescent children. Blood pressure, blood lipids, body fatness, and behavioral risk factors are being assessed. The intervention includes curriculum, school environment, and family components. After an initial 3-year pilot phase, the study is now in the fourth year of a 4-year trial.

THIS RESEARCH PROJECT IS FINISHED, PLEASE DISCONTINUE

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

To compare the effectiveness of curriculum plus school plus family intervention components versus curriculum plus school versus measurement only in modifying blood pressure, blood lipids, diet and physical activity.

To test for associations between independent variables such as psychosocial factors and behavior change.

To test the extent and quality of implementation and test for associations between implementation and outcomes.

Test the reliability and validity of the Physical Activity Interview with children.

Methods Employed:

In each of four study sites, 24 schools have been randomized to one of three treatment conditions: measurement only, curriculum and school environment intervention components, or curriculum, school environment, and family intervention components. Approximately 2400 third-grade children in each site (total N=9600) have been recruited into the study. During Fall 1992, each subject providing individual and parental permission completed assessments at school that included blood pressure, blood lipids, body composition, diet and physical activity. During class students completed a health behavior and psychosocial questionnaire. In Spring 1992, intervention began and will continue through 1994 when the cohort is in fifth grade. Final assessments will take place in the Spring of 1995.

Significance to Biomedical Research and the Program of the Institute:

This study will demonstrate the effectiveness of state-of-the-art school-based interventions in terms of behavior and physiological change. The study has potential to provide direction for school-based practice and policy regarding health education in schools. Validation of the PAI will provide a useful instrument for assessing physical activity in school-aged children.

Proposed Course:

The study, which began in April 1991, will run until the Spring of 1995. Intervention began in Spring 1992, and will continue through Fall 1994. During Spring 1995, post assessments will be conducted. Analyses of the baseline data are ongoing.

Publications:

Armstrong N, Simons-Morton BG. Physical activity and blood lipids in adolescents. Ped Exerc Sci 1994;6:381-405.

Simons-Morton BG, et al. Physical activity of preadolescent children: Baseline from the Children and Adolescent Trial for Cardiovascular Health (CATCH). Am J Public Health, in press.

Simons-Morton BG, Taylor WC, Huang IW. Validity of the physical activity interview with preadolescent children. Res Q Exerc Sport 1994;65:84-8.

Turley KR, Wilmore JH, Simons-Morton BG, Williston BG, Reeds J, Dahlstrom G. The reliability and validity of the 9-minute run in third grade children. Ped Exerc Sci 1994;6:178-87.

Contract Report:

No contracts issued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2108-04 PRB

PERIOD COVERED

October 1, 1994 through July 1, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Afro-American Adolescent Girls Growth and Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Center for Health Promotion Research, School of Public Health, University of Texas Health Science Center at Houston (W. Taylor)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.04	0.04	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of the study is to assess longitudinally among Afro-American girls the pattern and determinants of body weight, body composition, and aerobic fitness, and physical activity.

THIS RESEARCH PROJECT IS FINISHED, PLEASE DISCONTINUE

Project Description

Objectives:

Describe the patterns of weight, fatness, body composition, physical fitness and physical activity among the subject.

Test for associations between weight, fatness, and body composition, and physical fitness, physical activity and psychosocial variables.

Methods Employed:

The cohort includes the entire class of 6th grade students at one elementary school in Houston, Texas. Assessments are conducted three times annually, including a treadmill test of aerobic fitness, Tanner staging of maturational status, height, weight, body composition, diet and physical activity.

Significance to Biomedical Research and the Program of the Institute:

Afro-American girls are an understudied population. Overweight is a serious problem in this population and data indicate that fitness and physical activity decline among girls during adolescence. This study will examine these patterns and test for associations with behavioral and psychosocial factors.

Proposed Course:

This study is in its fourth year, funded by the NHLBI.

Publications:

None

Contracts:

No contracts issued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2109-04 PRB

PERIOD COVERED

October 1, 1994 through April 1, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

How Much Physical Activity Do Children Obtain During Physical Education Classes?

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: B. Simons-Morton Behavioral Scientist PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Center for Health Promotion Research, School of Public Health, University of Texas Health Science Center at Houston (W. Taylor)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research is to determine the extent to which physical education in a regional sample of elementary and middle schools provides students with moderate to vigorous physical activity during PE classes.

THIS RESEARCH PROJECT IS FINISHED, PLEASE DISCONTINUE

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

1. Determine the percent of physical education class time children and youth engage in moderate to vigorous physical activity (MVPA).

2. Assess association between the amount of MVPA and school and teacher variables such as class management and type of activity.

Methods Employed:

A sample of 20 elementary and seven middle schools from among 355 elementary schools and 117 middle schools in 20 school districts in Harris County, TX. Seven classes at each school were randomly selected and observed according to a standard protocol. Observers used a direct entry system to enter data directly into a hand held computer.

Major Findings:

1. Elementary school's students were observed to spend an average of 8.7% of class time in moderate to vigorous physical activity.

2. Middle school students were observed to spend an average of 16.1% of class time in moderate to vigorous physical activity.

Significance to Biomedical Research and the Program of the Institute:

2000 National Health Objectives call for an increase in the percent of PE time devoted to MVPA. Research is needed that documents the status of PE in terms of the amount of time devoted to MVPA.

Proposed Course:

This research has been completed, one paper has been published, and a second paper is under review.

Publications:

Simons-Morton BG, Taylor W, Snider SA, Huang IW. The physical activity of fifth-grade students during physical education classes. Am J Public Health 1993;83:262-4.

Contracts: No contracts issued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2110-04 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Preventing Problem Behavior Among Middle School Students

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
Others:	L.A. Maiman	Chief	PRB, DESPR, NICHD
	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	D. Haynie	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Behavior and Health Research, Inc. (K. Saylor)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of the study is to develop, implement, and test the efficacy of a multi-component program of school-based interventions in preventing problem behavior in a sample of middle school students. Interventions would be directed at the students to improve their resistance to problem behavior and toward the school environment and parent behavior to provide support for student prosocial behavior. The primary research question is, "What is the effect on the frequency of problem behavior among students participating in a special intervention that includes an orientation to middle school, nontraditional curriculum, and parent intervention compared to students in the comparison group?"

Project Description

Objectives:

1. Evaluate the extent and quality of implementation of the intervention components.
2. Evaluate the impact of the intervention on selected mediating variables, including student and parent self efficacy, expectancies, and perceived social norms.
3. Evaluate the impact of the intervention on the extent of student problem behavior.

Methods Employed:

Measurement: Baseline survey of problem behavior and hypothesized mediators.
Intervention: Curriculum, school environment, and family education.
Design: Six schools will be randomized to special intervention or usual care. Two cohorts of 6th graders (n = 2600) will be followed to 9th grade.

Major findings: None yet

Significance to Biomedical Research and the Program of the Institute:

Problem behavior is an important health problem among adolescents. The mission of the PRB is to conduct intervention studies to improve health and change health behavior of children and adolescents. This trial will test the efficacy of a multi-component program on problem behavior. Future studies can test the effectiveness of the components.

Proposed Course:

This year the contract was awarded to BHR, Inc., the interventions were developed, and the OMB application was completed.

Publications:

None

Contract Report:

Behavior and Health Research, Inc.
Contract No. N01-HD-4-3207
Title: Preventing Problem Behavior Among Middle School Students
Current funding: $495,150
Objectives: Develop and test multicomponent, school-based interventions to prevent problem behavior.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2114-03 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

MD/Family Partnership: Education in Asthma Self-Management

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, University of Michigan (N.M. Clark, D.W. Roloff, M.E. Hurwitz, M.A. Schork) and School of Medicine, Columbia University (R.B. Mellins, D. Evans)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.20	0.20	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major purpose of the MD/Family Partnership: Education in Asthma Self-Management study is to develop and test an intervention based on theories of self-regulation of behavior in changing the diagnosis and treatment patterns and educational behavior of physicians who treat children with asthma.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

It is hypothesized that education for physicians, based on the principle of self-regulation provided through an interactive seminar, will be effective in changing physicians' clinical behavior and improving their performance as health educators and counselors. The study also assesses the effects of education for pediatricians on the self-management behavior, health status, and health care utilization of their patients.

This research builds on an earlier study funded by the Agency for Health Care Policy and Research and NICHD to Dr. Maiman at the time she was a faculty member at the Department of Pediatrics, University of Rochester School of Medicine and Dentistry. Dr. Clark is Principal Investigator on the current study and funding is from NHLBI, NIH.

Methods Employed:

A randomized experimental design involving 60 pediatricians and 800 parent-child pairs from their practices will be employed. Data will be collected in Ann Arbor, Michigan and New York City. The first stage has been devoted to the development of the interactive seminar and video vignettes; development and field testing of physician and parent data collection instruments; and recruitment of study physicians. Baseline information has been collected from 58 physicians and 789 parent-child pairs, and 28 physicians attended the educational seminars. The first wave of follow-up data has been collected from both physicians and parents.

Significance to Biomedical Research and the Program of the Institute:

A strong partnership between physicians and patients has been discussed as a crucial part of effective asthma management. The study is unique in that it is the first evaluation research to focus on pediatricians who treat asthma and to apply theories of self-regulation of behavior to physician education. It is one of few to use changes in patient self-management behavior and health care use as outcomes of physician education. Findings will be important for the design of physician education related to asthma self-management and the strengthening of the partnership.

Proposed Course:

The study will be conducted over a five year period, funded by NHLBI. Baseline data are currently being analyzed. The next stage will be the implementation of the follow-up data collection, from health care utilization sources, parents and physicians.

Publications:

Clark NM, Nothwehr F, Gong M, Maiman LA, Hurwitz ME, Evans D, Roloff DW, Mellins RB. Enhancing physician-patient management of chronic disease: Elements of partnership. Acad Med, in press.

A paper ("The physicians' asthma counseling scale") was presented at the American Academy of Allergy and Immunology and American Thoracic Society Joint Conference in Chicago, IL.

Contract:

No contracts will be issued for the study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2115-03 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunization Among D.C. Infants

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
Others:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Howard University, DC General Hospital, Georgetown University Medical Center, Children's Hospital, DC Commission of Public Health, University of DC, Research Triangle Institute

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.30	0.30	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research is to assess the determinants of non-immunization among DC infants and young children.

PHS 6040 (Rev. 5/92)

Project Description

Objectives: In a sample of low-income, high risk DC mothers of children less than two years of age, identify the determinants of not initiating immunization and failure to maintain immunization status up to date.

Methods Employed:

The research will be conducted over a two-year period, beginning during the 1994-95 fiscal year. 480 mothers will be recruited to an interview after birth in the hospital and at home at 4 and 7 months post-partum.

Significance to Biomedical Research and the Program of the Institute:

Non-immunization during the first two years of life is an important health problem that has been understudied in the U.S.

Proposed Course:

The purpose of this prospective research is to identify predictors of lack of age-appropriate immunization and well child care among D.C. infants and children. At three D.C. hospitals, Howard University Hospital, D.C. General Hospital, and Washington Hospital Center, a cohort of 480 mothers will be recruited to be interviewed in the hospital at birth, and at home or by telephone at 3 months, and 7 months after birth. Assurance of age-appropriate immunization is a national public health priority. Unfortunately, the rates of age appropriate immunizations by age two average about 50% in U.S. cities, and less than 40% in the District of Columbia. Immunization against disease is an important component of pediatric primary health care and serves as an international marker of population health status. Attitudes toward immunization among low income mothers appear to be positive, however, participation in well child care, where most immunization occurs, is inadequate. Only one prospective study has been conducted in the U.S., and the causes of non-participation in well child care and low rates of immunization are largely unknown. The findings from this study will assist policy makers and public health officials in their efforts to increase rates of immunization and improve participation in well child care.

Publications:

None

Contracts:

No contracts are to be issued on this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2116-03 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Preventing Pregnancies Among Adolescents in the District of Columbia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
Others:	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	D. Haynie	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Howard University Medical Center, Georgetown University Medical Center, Children's Hospital, DC Commission of Public Health, DC General Hospital

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.55	0.55	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research is to test the efficacy of an intervention to prevent adolescent pregnancies. In concert with this effort, a preliminary study involving the collection and analysis of focus group data is being conducted to 1) develop an understanding of the perspectives, experiences, and motivations of Washington, D.C. youth with regard to sex, contraception, and pregnancy 2) obtain ideas for and reactions to intervention methods and content and 3) determine the extent to which existing theoretical and programmatic knowledge of teen pregnancy will be applicable to teens in Washington, D.C.

Project Description

Objectives:

In a sample of high risk DC adolescent girls:

1. Delay the time of initiation of sexual intercourse;
2. Prevent/delay initial and subsequent unintended pregnancies;
3. Prevent subsequent abortions.

Methods Employed:

Subjects will be recruited from public schools in the District of Columbia and randomized to special intervention or usual care conditions. Interventions include an educational curriculum and adjunct, school-based activities. Measures include sexual behavior, contraceptive use, pregnancy status, STDS, and other measures. The research will be conducted over a two year period, beginning September 1995.

Major Findings:

Although there are no findings currently from the larger study, preliminary findings from the focus group research are available. These results are based on the analysis of four groups of female, African-American clinic attenders (n=26). Sexual activity is viewed as the norm among these girls and their peers, and many of the girls initiate sex relatively young (age 13). Pressure to have sex originates from both boys and other girls. Participants perceive that they have control over whether or not sexual encounters will take place, but admit that other girls may not.

As one might expect, the population of clinic attenders is very aware of contraceptive methods. However, discussions suggest that participants are not entirely confident in the effectiveness of contraceptive methods. In addition, concern about the side effects of medical contraceptives make these options less desirable. Condom use is endorsed by most girls.

Pregnancy and child rearing are thought of as difficult and undesirable, yet not catastrophic. Participants frequently spoke of friends, relatives, and acquaintances who were teen parents. In the context of the discussions, participants were more concerned about preparedness of a girl for parenting and the quality of parenting a girl could provide rather than the timing of the pregnancy for a teenager.

As far as intervention methods are concerned, peers were perceived as a credible source for information on reproductive health, provided they were knowledgeable and experienced. Girls were typically unhappy with the way sex education is covered in school. Girls explained that schools focus "too much on plumbing" and not enough on feelings and relationships. They were also concerned that some school personnel were either intrusive or uncomfortable sharing information.

Significance to Biomedical Research and the Program of the Institute:

Adolescent pregnancy is an important health problem in need of research. We propose to develop and test the efficacy of a culturally sensitive intervention to prevent unintended adolescent pregnancies.

Proposed Course:

The Phase I needs assessment has been approved and is nearly complete. Additional focus groups are planned for the coming months. Phase II has been approved, and preparations for the intervention are underway.

Publications:

None

Contracts:

No contract is to be issued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2117-02 PRB

PERIOD COVERED

October 1, 1994 through June 15, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Validity of the Physical Activity Interview and Caltrac Motion Sensor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute effiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD

COOPERATING UNITS *(if eny)*

Center for Health Promotion Research, School of Public Health, University of Texas Health Science Center at Houston (W. Taylor)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use stenderd unreduced type. Do not exceed the space provided.)*

The purpose of this study is to assess the validity of the Physical Activity Interviews (PAI) and Caltrac assessments of moderate and vigorous physical activity of 3rd- and 5th-grade children, compared with monitored heart rates.

THIS RESEARCH PROJECT IS FINISHED, PLEASE DISCONTINUE

Project Description

Objectives:

Methods Employed:

Subjects (n = 21 fifth graders, 27 third graders) wore heart rate monitors and Caltracs for an average of 12.5 hours one day and were interviewed the following morning by trained interviewers following a standardized protocol.

Major Findings:

Means (adjusted to 10 hours) for all subjects combined (n = 61) were 67.2 minutes of PAI-reported moderate and vigorous physical activity, 44.3 monitored minutes ≥180%RHR, and 23.4 ≥200%RHR. Caltrac counts were 70.3 for 5th-graders (n = 21) and 85.2 for 3rd-graders (n = 27). The Pearson product-moment and intraclass correlations between PAI-reported activity minutes and monitored minutes 180%RHR were .72 and .62 for fifth graders and .57 and .41 for third-graders. Correlations between PAI-reported minutes of activity and monitored minutes 200%RHR were significant for both 5th-graders and 3rd-graders. The Pearson product-moment correlations between Caltrac counts and heart rate was 0.55 for 5th- and 0.70 for 3rd-graders at 180%RHR and 0.62 for 5th- and 0.80 for 3rd-graders for 200%RHR. These data provide support for the validity of the Caltrac and the Physical Activity Interview as measures of moderate and vigorous physical activity with preadolescent children.

Significance to Biomedical Research and the Program of the Institute:

Basic research on children's physical activity. Knowledge about variability in physical activitiy will allow researchers to calculate accurate sample size and power calculations in studies with preadolescent children.

Proposed Course:

Completed.

Publications:

Simons-Morton BG, Taylor W, Huang I. Day-to-day variability in physical activity among third graders. Ped Exerc Sci, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2118-01 PRB

PERIOD COVERED

"Project cancelled before it was initiated"

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parent-Child Conflict During the Transition to Middle School

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
Co-PI:	D. Haynie	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
Others:	L.A. Maiman	Chief	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research investigates changes in parent-adolescent conflict during the transition to middle school.

THIS RESEARCH PROJECT WAS CANCELLED. IT NEVER STARTED.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2119-02 PRB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parenting Style and Adolescent Substance Use and Depressive Symptoms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Department of Preventive Medicine, School of Medicine, University of Southern California (J.L. Richardson, C.W. Dent) and School of Public Health, University of Illinois at Chicago (B.R. Flay)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.10	0.10	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research investigates the role of parental supervision and monitoring, parenting style, and parent-adolescent conflict in adolescent substance use and depressive symptoms.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The goal of this research is to investigate the relationship between parenting styles and practices, parent-adolescent conflict, and adolescent substance use and emotional problems. Two areas are of particular interest: a) The importance of parental supervision and monitoring in the prevention of adolescent problem behavior and adjustment problems, and b) The nature of the relationship between parent-adolescent conflict and adolescent problem behavior and depression.

Methods Employed:

The data were gathered in February 1988, as a part of a larger survey study of adolescent smoking and drug use. The sample consisted of 3993 ninth-grade students in Los Angeles County and San Diego County, representing public schools in six school districts. Trained data collectors provided instructions and administered the questionnaires, which took approximately 45 minutes to complete, to entire classes of students. Letters describing the study were sent to the parents of children in these classrooms.

Significance to Biomedical Research and the Program of the Institute:

Research on the effects of parenting practices has found that certain parenting styles constitute a risk factor for adolescent behavioral and emotional problems, including substance use and depressive symptoms. One parenting style, called "authoritative," which combines parental acceptance with firm behavioral control, is considered a protective factor against negative influences on an adolescent. Research in this area can be useful in formulating new intervention and preventive strategies.

Proposed Course:

The University of Southern California data are currently analyzed to address issues related to parenting styles, parent-adolescent conflict, adolescent substance use, and adolescent depression.

Publications:

None to date. One manuscript has been submitted for a publication and is now under revision for a resubmission. Another manuscript is in preparation.

Contract Report:

No contracts were employed for this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2120-01 PRB

PERIOD COVERED

May 2, 1995 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Survey of Parent Intervention Strategies to Prevent Adolescent Alcohol Use

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
OTHERS:	D. Haynie	IRTA Post-Doctoral Fellow	PRB, DESPR, NICHD
	A. Crump	IRTA Post-Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

University of Maryland, Department of Health Education, University of Maryland, College Park (K. Beck)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.45	.45	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research investigates the associations between adolescent alcohol use and parental monitoring practices. Telephone interviews will be conducted with randomly selected adolescents (ages 14-19) and their parents in Maryland. Parent monitoring behaviors, parents' perceptions of their adolescents' drinking behavior and adolescents' self reports of drinking behaviors will be assessed. Drinking behaviors include underage consumption of alcohol, drinking and driving and riding with a driver who has been drinking.

Project Description

Objectives:

Parental monitoring practices are thought to have an influence on the likelihood of adolescents engaging in problem behavior. The current study will investigate the associations between parental monitoring and adolescent alcohol consumption, drinking and driving, and riding with an intoxicated driver. Questions of particular interest include: 1) the concordance between parent and adolescent reports of parents' monitoring practices as related to adolescent behavior, 2) the relationship between parent monitoring and enforcement of rules regarding alcohol use and adolescents' self reports of use, 3) parents awareness of their adolescents drinking in relationship to their monitoring behaviors, and 4) age and/or gender differences in parenting practices regarding adolescent drinking.

Methods Employed:

A random telephone survey will be conducted with 900 parents and their adolescents in the state of Maryland.

Significance to Biomedical Research and the Program of the Institute:

Underage drinking and driving and driving pose significant risks to adolescent health and well being. Parental monitoring is one of many factors that is important for understanding and addressing this significant health problem. We seek to determine not only what parents say they do to monitor their adolescent children's behavior regarding drinking, but also to assess the extent of concordance between what parents say they do and adolescents report of their parents' monitoring behaviors and expectations for them regarding alcohol. Understanding current parenting practices in relation to adolescent behavior is an important step in designing effective interventions to address underage drinking and adolescent drinking and driving.

Proposed Course:

Data collection will begin in late September 1995, and will be completed by the end of November 1995. Data analyses will occur in early December.

Publications:

None

Contract Report:

Study is funded through a professional services contract awarded to Ken Beck, Ph.D., of the University of Maryland Department of Health Education (Purchase Order # 263-MD-526633).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2121-01 PRB

PERIOD COVERED

May 1, 1995 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parent-Child Interaction and Asthma Self-Management

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, University of Michigan (N.M. Clark); Department of Pediatrics, College of Physicians and Surgeons, Columbia University (D. Evans)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.30	0.30	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of the study is to determine if there is a relationship between the process of parent-child interaction regarding medically recommended self-management behaviors for asthma and the adoption of these behaviors by young adolescents, and, if the adoption of these behaviors influences the child's health status and health care utilization. The survey will be conducted as a "substudy" to an on-going investigation ("main study") of the effects of a physician training program on physician activity related to pediatric asthma-related patient education and adoption of self-management strategies. The subjects in the study are 293 asthmatic children, ages 8-13, and their parents, from two cities: Ann Arbor and New York.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The goal of the proposed study is to determine if parent-child interaction regarding asthma self-management influences the adoption of self-management behaviors by young adolescents with asthma, and, if the adoption of these behaviors influences their health status and health care utilization. The specific aims are to examine: 1) the process of collaboration between parents and young adolescents in solving asthma self-management problems; 2) parental behaviors for structuring and managing these interactions; 3) the processes involved in the transfer of responsibility from the parent to the child to manage asthma; 4) young adolescent's information-processing to adopt and follow self-management strategies; 5) geographical and ethnic differences in parent-child communication regarding asthma self-management; and 6) potential systems for delivery of health promotion education to parents and children.

Methods Employed:

The substudy survey will be an adjunct to the main study. Phase I, a unique data collection effort for the substudy, will employ face-to-face exploratory interviews and focus groups with both parents and children (N = 30 parent-child pairs) to provide information for the construction of items to be incorporated in the Phase II interviews. Phase II data collection (N = 293 parent-child pairs) will be aligned with the final follow-up telephone interviews of the main study. For parents, the substudy questions will be added to the parent interview. Data collection from children will be a unique effort for the substudy.

Significance to Biomedical Research and the Program of the Institute:

Information from this substudy will be used to help guide future efforts to educate both families and physicians in an attempt to improve the self-management process of parents and children in pediatric asthma and other pediatric conditions, increase the adoption of self-management behaviors by children, and improve child health status and reduce health care utilization.

Proposed Course:

Phase I (focus interviews) will be conducted from September 1995 through October 1995. Data collected at focus interviews will be processed from November 1995 through April 1996. Phase II (final telephone interviews) will be conducted from May 1996 through July 1996.

Publications: None to date.

Contract Report:

Professional Service Contract, awarded to Noreen M. Clark, School of Public Health, University of Michigan.

Purchase Order No. 263-MD-534897

Title: "Parent-Child Interaction to Promote Improved Health Status in Young Adolescents with Asthma."

Date Purchase Order No. was initiated: 7/17/95

Current funding: $24,840

Objectives: To examine the relationship between asthma-related interaction patterns between parents and children and the adoption of asthma self-management strategies by young adolescents.

Methods employed: Face-to-face exploratory interviews and telephone interviews.

Major findings: none to date

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2122-01 PRB

PERIOD COVERED

June 1, 1995 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
Others:	L. Maiman	Chief	PRB, DESPR, NICHD
	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, The Johns Hopkins University (C. Alexander)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.07	0.07	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The proposed study is a qualitative assessment of the perceptions and behaviors of parents of high risk youth in regard to the prevention of early initiation of intercourse and the negative outcomes associated with this behavior. This research also addresses the strategies parents use to influence their child's behavior and the barriers to implementing these strategies.

Project Description

Objectives:

Early adolescents who are sexually active are at risk of adverse outcomes associated with sexual intercourse. Compared to older youth, early adolescents have less access to information and services needed to prevent pregnancy and sexually transmitted diseases (STDs). In addition, younger teens are still developing cognitive skills that are needed to successfully utilize methods of pregnancy and STD prevention correctly and consistently. This research aims to explore parents' perceptions regarding the problems associated with sexuality among early adolescents and parents' ability to influence this behavior. The objectives of this research are as follows: 1) identify how parents perceive early onset of sexual activity and its potential outcomes 2) identify strategies parents utilize to control or influence sexuality related behaviors of their early adolescents, and 3) identify psychosocial and other barriers to parents' implementation of prevention strategies to control or influence sexuality related behaviors of their early adolescents.

Methods Employed:

Twenty-four parents of 13-14 year old females will participate in the study. These parents will be recruited through school-based health centers to participate in one to two in-depth interviews based on a semi-structured guide. The interviews will be tape recorded and transcribed. Data will be coded and analyzed, and this process will be facilitated by a text management program.

Significance to Biomedical Research and the Program of the Institute:

Twenty-three percent of U.S. adolescents have initiated sex by age fourteen, and in populations at high risk for early, unintended pregnancy, as many as half have initiated intercourse at this age. While most interventions to prevent the undesirable outcomes of sexual behavior focus on the adolescent, some recent interventions have attempted to involve parents. Yet little is understood about parents' perceptions of this behavior nor their perceived ability to assist their children to prevent adverse outcomes. Understanding parents' perceptions concerning early initiation of sex, STDs and pregnancy is critical to successfully involving them in preventive interventions. This research will provide qualitative information that is needed to generate hypotheses for a quantitative study of parents' perceptions and behaviors.

Proposed Course:

Proposal development began on June 1, 1995, and study design and interview construction will begin September 1, 1995. The data collection will begin December of 1995, and will be completed by February 1996. Analysis of the data will be complete by September 1996.

Publications:

None

Contract Report:

A professional services contract is in the process of being initiated with The Johns Hopkins University Center for Adolescent Health Promotion and Disease Prevention, under the direction of Cheryl Alexander, Ph.D. This contract, entitled "Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth," provides for the contractor to facilitate the data collection and analysis for this study. This includes recruiting the sample, scheduling interviews, arranging for data transcription, coding data, and participating in other data analysis functions. The NICHD investigators are responsible for conducting the interviews and directing the data analysis effort. The duration of the contract is one year, and it is funded for $21,149.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD00035-23 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Structure and Function of Peptides and Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H.C. Chen	Head, UMSPC	ERRB, NICHD
Others:	K.J.Catt	Chief	ERRB, NICHD
	J.L. Morell	Research Chemist	ERRB, NICHD
	L. Sun	IRTA Fellow	ERRB, NICHD

COOPERATING UNITS *(if any)*

Lab. of Biochemical Physiology, DCT, NCI (H-F. Kung); Department of Biochemistry, New York University School of Medicine (S. Lee-Huang)

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Unit of Molecular Structure and Protein Chemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project focuses on the design and synthesis of peptide antigens, the immunological characterization of peptide hormone receptors, structure-function relationship studies, determination of amino acid sequences, and the identification and characterization of post-translational modifications of proteins: (1) We obtained high-level expression of a 92 residue protein, designated as LE93 corresponding to the C-terminal sequence (residues 268-359) of the angiotensin II (AII) receptor type 1b in *E. coli* strain TB1 transfected with pMAL-2c vector consisting of the inserted gene down stream of the *mal*E maltose binding protein gene, and a protease Factor Xa site. The antiserum raised against this protein recognized a 66-67 kDa protein as the AII receptor with high specificity as judged from Western blot analyses of bovine adrenal plasma membrane and cells, and rat tissue homogenates, as well as immunoprecipitation of the radioiodinated [Sar-1,*p*-azidoPhe-8]AII photoaffinity labeled receptor from the same samples. (2) Nine peptides covering all putative cytoplasmic and extracellular domains of the murine GnRH receptor were synthesized, conjugated to bovine thyroglobulin, and the conjugates were used to raise antibodies in rabbits. (3) The gene encoding MAP30 (anti-HIV protein from *Momordica charantia)* has been cloned based on two sequences of tryptic fragments and used to express MAP 30 in *E. coli* using the expression vector pRSET. The recombinant protein exhibited identical anti-HIV and anti-tumor activities to those of the plant protein. (4) In addition to N-glycosidase activity on 28S ribosomal RNA and topoisomerase activity on plasmid and viral DNAs including HIV-1 long terminal repeats, MAP 30 and GAP 31 (anti-HIV protein from *Gelonium multiflorum*) were shown to inhibit 3'-processing, integration, and disintegration catalyzed by the HIV-1 integrase. These findings suggest that impediment of viral DNA integration may play a key role in the anti-HIV activity of these plant proteins. (5) A novel protein kinase (PK) C substrate, PP28 (Mr=28 kDa), has been purified from the heat- and acid-stable fraction of rat brain extract. Based on two segments of the N-terminal sequence, a cDNA encoding PP28 was cloned to express the protein in *E. coli* using the expression vector pRSET. It encodes a 180 amino acid sequence. Although both tissue and recombinant PP28 can be phosphorylated by PKA, PKC, and casein kinases(CK) I and II *in vitro,* only CK II appears to phosphorylate the protein in N1E115 neuroblastoma cells *in vivo*. The *in vitro* CK II phosphorylation of PP28 yielded phosphorylated Ser-62 and Ser-59 as predicted for CK II phosphorylation. The PP28 protein, which is widely distributed in tissues, may thus may be phosphorylated in a tissue-specific or in a highly regulated manner.

Project Description

Objectives:

This project is concerned with the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins with particular emphasis on the study of physico-chemical characteristics, and structural studies in order to elucidate different molecular forms and structural requirements for various biological activities. During this project year, research has focused on the design and synthesis of peptide antigens, the immunological characterization of peptide hormone receptors, structure-function relationship studies, determination of amino acid sequence, and the identification and characterization of post-translational modifications of proteins.

Methods Employed:

A wide variety of advanced techniques for peptide and protein chemistry were employed. These include amino acid microanalysis, protein sequencing, peptide synthesis, electrophoresis, spectropolarimetry, and conventional and high performance liquid chromatography. Special methods using novel protecting groups to meet the requirement of highly selective removal in peptide synthesis and specific chemical techniques for the modification and cleavage of amino acids in a peptide chain were designed and employed. A statistical computer program was used for the calculation of α-helical and ß-sheet structures in peptides. Specific methods employed included PCR, Western blot analysis, immunocytochemical analysis, *in situ* hybridization, ribonuclease protection assay, and photoaffinity labeling of receptors.

Major Findings:

Expression of angiotensin II receptor segments and generation of antibodies. Based on the sequence of the rat angiotensin II (AII) type 1b (AT_{1b}) receptor, eight segments from different putative domains in the receptor protein were selected for chemical synthesis by the solid phase method. The peptides were conjugated with thyroglobulin or keyhole limpet hemocyanin. Rabbits immunized with these conjugates produced appreciable antibody titers. However, the antibodies are of low avidity and were not sufficiently specific for the immunological characterization of the receptor protein. In order to generate antibodies of improved avidity, a rational approach by increasing the size of antigen was employed. Since several attempts to over-express AII receptors have not been successful here and elsewhere, we therefore undertook an approach to express the protein in segments consisting of extracellular, transmembrane, and cytoplasmic domains. We have obtained mg quantities of the protein corresponding to the amino acid residues 265-359 of the AT_{1b} (designated as LE92) receptor expressed in *E. coli* strain TB1 transfected with a plasmid with the corresponding gene inserted downstream from the *mal*E gene in the pMAL-2c (Biolabs) vector. This construct also contains the sequence coding for the recognition site of the specific protease Factor Xa located at the 5' insertion site. The *mal*E gene encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein which allows a one-step purification through maltose-affinity chromatography. Upon purification of LE92/MBP conjugate from cell lysate by maltose affinity chromatography and cleavage of the conjugate with Factor Xa, a protein of 13.5 kDa on SDS-PAGE was obtained which was recognized by antibodies generated against the C-terminal peptide of AT_{1b} (residues 330-359) by Western blot analysis, and yielded an N-terminal amino

acid sequence consistent with LE92. For the generation of antibodies, the LE92/MBP fusion protein was used to immunize rabbits.

Immunochemical characterization and molecular weight determination of angiotensin II receptor in tissues and cells. The antibodies against LE92/MBP (AT_{1b} receptor, Res.265-359 maltose binding protein conjugate) permitted characterization of AII receptors by the Western blot analysis using either enhanced chemiluminescence or ^{125}I-Protein A monitoring. A band with molecular size of Mr=66-67 kDa was found in a bovine adrenal membrane preparation and adrenal cells. A minor band at 44-45 kDa which was detected by anti-peptide antibodies in previous studies was no longer present. Similarly, the band at Mr=66-67 kDa was found in rat tissue homogenates in which adrenal tissue showed the highest intensity followed by brain, uterus, liver, lung and kidney. Unlike previous studies with anti-peptide antisera, no 66-67 kDa band was found in heart and spleen. Photoaffinity labeling of bovine adrenal membrane and tissue homogenates with ^{125}I-[Sar^1,(4-N_3)Phe^8]AII followed by immunoprecipitation using the antiserum against LE92 and SDS-polyacrylamide gel electrophoresis revealed that only a major radioactive band corresponding to 66-67 kDa was detected from the immunoprecipitates. The presence of 10^{-5}M unlabelled AII completely blocked the cross-linking. The amount of receptor in various tissues estimated by the intensity of radioactivity cross-linked ligand was consistent with the radio-ligand binding data. Together with these data from Western blot analyses, it appears that the AII receptor has a molecular weight of 66-67 kDa.

Chemical synthesis of GnRH receptor segments. Based on the deduced sequence of the murine gonadotropin-releasing hormone (GnRH) receptor, seven segments from different domains in the receptor protein sequence were selected and synthesized by the solid phase method. They were designated as GnRH1/12 (Res. 316-327, in seventh transmembrane domain and C-terminal), GnRH2/19 (Res. 248-266, in third cytoplasmic domain) GnRH3/22 (Res. 14-35, first extracellular domain), GnRH4/42 (Res. 229-270, all third cytoplasmic domain), GnRH5/20 (Res. 178-197 in third extracellular domain), GnRH6/16 (Res. 291-306, fourth extracellular domain) and GnRH7/30, Res. 105-121 & 186-198, the second and third extracellular domains cross-linked between two inner Cys residues). Two more peptides from the cytoplasmic domain were also synthesized, and were designated as GnRH8/25 (Res. 58-82) and GnRH9/25 (Res. 134-158), respectively. The peptides were conjugated to thyroglobulin through Sulfo-SMCC, a heterobifunctional linker, via added cysteine on the N-terminus of the peptide. Rabbits immunized with thyroglobulin conjugates of these peptides produced appreciable titers of antibodies. Characterization and affinity purification of these antibodies are also in progress.

Expression and characterization of recombinant anti-human immunodeficiency virus (HIV) protein from bitter melon. Previous reports have already indicated purification and characterization of new class of anti-HIV plant proteins: MAP 30, TAP 29, GAP 31, DAP 32 and 30. These single chain ribosome-inactivating proteins inhibit HIV-1 infection as well as replication, as measured by syncytium formation and viral core protein p24 expression and HIV-associated reverse transcriptase. All of these plant proteins demonstrate dose-dependent antiviral potency with undetectable cytotoxicity in host cells or insignificant toxicity in mice. Based on the N-terminal sequence of MAP 30 , Asp-Val-Asn-Phe-Asp and a tryptic fragment, Lys-Gly-Asn-Ile-Lys presumably residing near the C-terminus as well as sequence homologies to Map 30II determined previously in this laboratory, oligonucleotides primers were synthesized for PCR amplification to generate probes for the screening of bitter melon genomic library. The gene encoding MAP30 has been cloned and used to express MAP30 in *E. coli* using the expression vector pRSET. The gene encoding MAP30 contains no intron. It codes 286 amino acid sequence including a 23 amino acid leader. The purified recombinant MAP30 showed a slightly smaller size than

the natural MAP30 presumably due to the lack of glycosylation in *E. coli*. Nevertheless, the natural and recombinant proteins exhibit identical anti-HIV and anti-tumor activities.

Inhibition of Human Immunodeficiency virus Type I integrase by MAP 30 and GAP 31. Both MAP 30 and GAP 31 have been established to exhibit N-glycosidase activity on 28S ribosomal RNA and a topological activity on plasmid and viral DNAs including HIV-1 long terminal repeats (LTR) in our previous studies. LTRs are essential sites for integration of viral DNA into the host genome by viral integrase. The dose-dependent inhibition of MAP 30 and GAP 31 on HIV-1 integrase has also been demonstrated for the first time. Inhibition was studied using oligonucleotide substrates with sequences corresponding to the U3 and U5 regions of HIV-LTR All of the three specific reactions catalyzed by the integrase, namely, 3'-processing (specific cleavage of the dinucleotide GT), strand transfer (integration) and disintegration were inhibited. In the presence of 20 ng viral substrate, 25-50 ng target substrate, and 4 μM integrase, total inhibition was achieved at equimolar concentrations of the integrase and the antiviral proteins with EC_{50} of approximately 1 μM. Integration of viral DNA into the host chromosome is a vital step in the replicate cycle of retroviruses including the AIDS virus. The inhibition of HIV-1 integrase by MAP 30 and GAP 31 suggests that impediment of viral DNA integration may play a key role in the anti-HIV activity of these plant proteins.

Molecular cloning and biochemical characterization of 28 kDa acidic protein from rat brain. In a collaboration with the Section on Metabolic Regulation, ERRB, on the search for novel protein kinase (PK) C substrates, a protein (Mr=28 kDa), designated as PP28, has been identified and purified from the heat- and acid-stable fraction of rat brain extract. The N-terminal sequence analysis yielded 40 residues that did not correspond to any known proteins in the Swissprot data bank. Based on the sequences RKGGHKG (residues 5-11) and NEEDEQQ (residues 34-40), forward and reverse nucleotide primers were synthesized, respectively. The gene encoding PP28 was cloned to express the protein in *E. coli* using the expression vector pRSET. It encodes a 180 amino acid sequence which gives a calculated mol. wt. of 20,491 but migrates on SDS-PAGE as 28 kDa as well as having predicted phosphorylation sites for PKC, casein kinase (CK) I and II and PKA, but no consensus Asn-glycosylation site. The larger apparent mol. wt. on SDS-PAGE is attributable to its being extremely rich in hydrophilic amino acids and perhaps nonglobular through lacking intra-chain disulfide linkages. Although both tissue and recombinant pp28 can be phosphorylated by the aforementioned kinases *in vitro* and show different ^{32}P-labeled tryptic peptide patterns, *in vivo* phosphorylated PP28 in N1E115 neuroblastoma cells showed only a pattern identical to that obtainable by CK II phosphorylation. Ser^{62} as the major and Ser^{59} as the minor phosphorylation site consistent with CK II phosphorylation were determined by amino acid sequence analysis. It appears that CK II is solely responsible for the phosphorylation of PP28 *in vivo*. The PP28 is widely distributed in tissues as revealed by Northern blot analysis. In addition to brain, which appears to be the most abundant source, appreciable amounts were found in lung, spleen, kidney, liver, heart, and muscle. The role of PP28 and its regulation by phosphorylation are still under investigation.

Significance to Biomedical Research and the Program of the Institute:

We have obtained a new antiserum generated against a 92 residue C-terminal segment of angiotensin (AII) receptor type 1b that recognizes by the Western blot analysis a 66-67 kDa protein in bovine adrenal plasma membrane and cells, rat tissue homogenates of adrenal, liver, brain and uterus but not heart or spleen. This finding is consistent with the data from AII binding experiments. The photoaffinity labeling of bovine adrenal plasma membrane and rat tissue homogenates with ^{125}I-[Sar^{1},(4-N$_3$)Phe^{8}]AII and immunoprecipitation with this antiserum revealed also a 66-67 kDa species. Previously, Western blot

analyses using six antisera generated against peptides representing various cytoplasmic and extracellular domains of the receptor detected 66-67 and 44-45 kDa species of AII receptors in both bovine adrenal plasma membrane and cells. The same two species were also found in the homogenates of rat heart and spleen in which no AII binding was detected. Nevertheless, the affinity labeling and immunoprecipitation experiments using the peptide antisera revealed only 66-67 kDa species. Collectively, the new antiserum raised against LE92/MPB recognizes AII receptor type 1 with improved specificity. This antiserum will be useful for immunocytochemical study of the receptor localization in tissues, and for Western blot analysis for the expression and biochemical characterization of AII receptors. Furthermore, several antisera will be screened for their ability to inhibit AII binding to their respective receptors and the study of subsequent biochemical responses.

GnRH plays a pivotal role in LH and FSH secretion of the pituitary gland. Determination of the structure of the GnRH receptor may lead to an understanding of the molecular mechanism of receptor activation and regulation. Although the amino acid sequence deduced from GnRH cDNA suggests seven putative transmembrane domains characteristic of G-protein-coupled receptors, it allows the assignment of a very short intracellular C-terminal segment lacking a typically polar sequence. Antisera against various domains of the receptor protein will be essential for immunocytochemical study of the receptor localization in tissues, and for Western blot analysis for the expression and biochemical characterization of the GnRH receptor. We synthesized these peptides also for the purpose of investigating of their ability to compete with the receptor for GnRH mediated G-protein coupling and protein kinase activation.

Studies of compounds derived from medicinal plants have yielded basic knowledge that is critical to modern medicine. A series of proteins designated as MAP 30, MAP 30II, TAP 29, GAP 31, DAP 30 and DAP 32 have been isolated and characterized. The discovery that MAP30 and GAP31 are potent inhibitors of HIV-1 integrase suggests that these antiviral agents act on a critical target indispensable to the life cycle of the AIDS virus. Previously, we have identified two activities that may be critical to the antiviral action of these proteins. The first is N-glycosidase activity that cleaves the specific glycosidic linkage between the adenine and the ribose at A^{4324} and G^{4323} on the 28S rRNA and thus inhibits protein synthesis. The second is a DNA topological inactivation that renders viral and plasmid DNA topologically inactive. It is important to define the extent to which these activities or other activities yet to be identified contribute to anti-HIV activity. These studies should provide basic insights on how to enhance anti-HIV activity while eliminating other activities that do not contribute to anti-HIV function. The ongoing studies of the three dimensional structures of these proteins may lead to the identification of motifs required for various activities. In particular, a rational design can be performed in order to obtain proteins that may display high activity and therapeutic index against infection and replication of HIV. The cloning of the MAP30 gene and the expression of biological active recombinant MAP30 should also facilitate clinical development of this antiviral and anti-tumor agent as well as its site-specific modulation to develop more effective anti-HIV drugs.

The pp28 is a novel phosphoprotein from rat brain. Although it was first known as a PKC substrate *in vitro* and contains consensus phosphorylation sites for CK I, CK II and PKA in addition to PKC, only two sites corresponding to the consensus sites for CK II were found to be phosphorylated in N1E115 neuroblastoma cells *in vivo*. Furthermore, the protein is widely distributed in tissues. Thus this protein may be phosphorylated in a tissue specific or in a highly regulated manner.

Proposed Course:

We will continue our efforts on the purification and characterization of antibodies specific to either AII or GnRH receptors, and studies on the tissue distribution, expression and inhibition of glycosylation and signal transduction of these two receptors. Tissue distribution and expression of these two receptors will be studied in fetal, infant, and adult rats under the influence of various physiological and pathophysiological conditions. Purification of receptor proteins using immunoaffinity chromatography and their biochemical characterization will be pursued. Attempts will be made to achieve high-level expression of angiotensin II receptor as the whole protein or its large fragments comprised of three domains in bacteria and baculovirus systems. The whole open reading frame and a partial sequence of AT_{1b} DNA have been cloned into these two fusion protein expression systems. Although some difficulty will be encountered in order to express a protein with seven transmembrane domains, over-expression of a partial sequence with one or two transmembrane, extracellular and cytoplasmic domains may be feasible. Expressed proteins will be used to immunize rabbits to generate specific polyclonal antibodies to study their biochemical characteristics.

Efforts will also be focused on the structural characterization of phosphoproteins isolated from tissues and expressed in transfected cells. Structure and function relationships will be investigated. The search for new protein kinase substrates will continue by performing sequence analyses of a group of heat- and acid-stable proteins of 10-20 kDa from the rat brain cytosolic fraction.

Publications:

Lee-Huang S, Kung HF, Huang Paul, Bourinbaiar AS, Morell JL, Brown JH, Huang PhL, Tsai WP, Chen AY, Huang HI, Chen HC. HIV-1 inhibition, DNA binding, RNA binding and ribosome inactivation activities in the N-terminal segments of the plant anti-HIV protein GAP 31. Proc Natl Acad Sci USA 1994; 91:12208-12212.

Lee-Huang S, Huang PL, Chen HC, Huang PL, Bourinbaiar AS, Huang HI and Kung SF. Anti-HIV and anti-tumor activities of recombinant MAP 30 from bitter melon. Gene 1995; 161:151-156.

Lee-Huang S, Huang PL, Huang PL, Bourinbaiar AS, Chen HC, and Kung SF. Inhibition of human immunodeficiency virus type 1 (HIV-1) integrase by anti-HIV plant proteins MAP30 and GAP31. Prod. Natl Acad Sci USA (in press).

Ryback SM, Lin JJ, Newton DL, Kung SF, Monks A, Chen HC, Huang PL, Lee-Huang S. *In vitro* anti-tumor activity of the plant ribosome inactivating proteins MAP 30 and GAP 31. Int'l J Oncol 1994; 5:1171-1176.

Seki K, Chen HC, Huang KP. Dephosphorylation of protein kinase C substrates, neurogranin, neuromodulin and MARCKS by calcineurin. Arch Biochem Biophys 1995; 316:673-679.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00146-19 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Studies of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H.C. Chen Head, UMSPC, ERRB, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Unit of Molecular Structure and Protein Chemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00147-19ERRB

PERIOD COVERED October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism of Action of Peptide Hormones in Steriodogenic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: M.L. Dufau Chief, SME, ERRB, NICHD

COOPERATING UNITS *(if any)*

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SECTION
Section on Molecular Endocrinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00150-20-ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Gonadal Receptors and Mechanisms of Action of Peptide Hormones in Steroidogenic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Maria L. Dufau	Chief, SME	ERRB, NICHD
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COOPERATING UNITS *(if any)*

University of Rome "La Sapienza; Department of Medicine, University of Virginia, Charlottesville
Contract for preparation of gonadal cells and cell fractions HD-6-2904

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Section on Molecular Endocrinology

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1) Overexpression of the LH holoreceptor and extracellular hormone-binding spliced variant form B was achieved in insect cells. Overexpressed receptors are glycosylated (high mannose) and exhibit high-affinity hormone binding, these are amenable for structural studies. 2) Functional CHO chains required for hormone binding were at Asn-173 and Asn 152 and each mannose chain contributed about 2kDa. Asn-77 was not glycosylated but its mutation to Gln reduced hormone binding reflecting its location in a crucial region of the binding domain. The proximal N-acetylglucosamine linked Asn, common to the mannose (insect) and complex (mammalian) chain, is the functional sugar moiety that is necessary for the acquisition of the high-affinity conformation. This sugar residue is essential for refolding of the denatured and reduced native receptor and possibly of the nascent receptor LHR. 3) Gene transcription of LH receptor is dependent on Sp1-induced promoter activation from two of four Sp1 binding domains Sp1-2 (canonical) and Sp1-4 (non-canonical) that contributes equally to transcriptional initiation. The Sp1-4 binding site within $Sp1_4$ was identified as 5' GGG GTG GGG rather than to the 3' overlapping G-C box. Functional non-Sp1 protein(s) bind the 3' GC box and only in non-expressing cells are functional substitute for the downstream M1 regulatory domain. Removal of both transfactors may play a role in permanent silencing expression. 4) Analyses of the 5' UTRs of the prolactin receptor (PRLR) mRNAs expressed in gonadal and non-gonadal tissues, and their genomic organization, revealed three alternate first exons. Each of these exons is spliced to a common exon 2 that precedes the third exon containing the translation initiation codon. Alternate utilization of these first exons, and alternative splicing of exon 2, generate multiple 5' UTRs in PRLR transcripts. Three functional PRLR gene promoters that initiate the transcription were identified. These were found to be utilized in a tissue-specific manner both *in vivo* and *in vitro*. These findings indicate that multiple promoters control transcription of the PRLR gene, and provide a molecular basis for the differential regulation of PRLR expression in diverse tissues. 5) An homology model of the rat CY17 was derived from the alpha/betaF supersecondary structural element of the 3alpha/20beta hydroxysteroid dehydrogenase of *Streptomyces Hydrogenans*. 6) GHRH present in the Leydig cell is under positive control of LH and synergyzes FSH action in Sertoli cells. GHRH is also localized to the acrosomal region of early to intermediate spermatids. GHRH from Leydig and germinal cell origin may be a paracrine regulator of Sertoli cells and exert intracrine functions in the spermatids.

Project Description:

Objectives:

A. Gonadal receptors: structure, regulation, and function.

(1) Investigation of physical, molecular and functional characteristics of gonadotropin and prolactin receptors, including gene structure, expression, and translation; (2) Overexpression of LH and FSH receptors, and development of scale-up procedures for purification of receptors for structural analysis; (3) Analysis of the relationship between receptor sites and adenylate cyclase enzyme complex; and (4) Investigation of membrane protein phosphorylations as intermediate steps in hormone action.

B. The androgen pathway: hormonal regulation of testicular function.

(1) To define the mechanisms of action of trophic hormones upon steroidogenesis and in the broadest sense the modulatory action of peptide hormones and growth factors upon development and functions of the Leydig cell. (2) To study cell-to-cell communication within the interstitium and the functional interactions of the Leydig cell with the tubule compartment and *vice versa*.

Methods Employed:

A. Gonadal receptors: structure, function, and regulation.

Assay of particulate and soluble receptors by ligand-binding and centrifugation, millipore filtration and polyethylene glycol precipitation; adenylate cyclase assay; affinity chromatography; SDS gel electrophoresis. Electroblotting; double dimension gel electrophoresis. Phosphorylation of membrane proteins and purified receptors. Cholera toxin and pertussis toxin-catalyzed ADP ribosylation. Immunization of mice and rabbits with purified receptors; fusion, screening, production of monoclonal and polyclonal antibodies to receptors; microsequence analysis of lactogen and LH receptor N-terminal regions and protein fragments. Peptide synthesis, DNA synthesis, construction of DNA probes, screening genomic libraries, cloning procedures, DNA sequencing. Subcloning of receptor cDNA, wild type receptor fragments and mutated forms in expression vectors. Expression of receptors in COS cells, Y_1 cells, and CHO and HeLa cells. Nb_2 lymphoma cell culture, bioassay of lactogenic hormones. Overexpression of receptors in insect SF9 and high-five cells. Site-directed mutagenesis of cDNA and genomic fragments by RCPCR. Subcloning of 5' and 3' flanking regions of the LH and prolactin receptor genes in CAT vector and Luciferase vectors. CAT and Luciferase assays, gel shift analyses and foot printing. Isolation of specific transacting factors; nuclear transcription assays.

B. The androgen pathway: hormonal regulation of testicular function.

Preparation of Leydig cells by collagenase digestion. Purification of Leydig cells by centrifugal elutriation and metrizamide gradients. Electron microscopy, light microscopy and autoradiography. Characterization of LH, GnRH, CRF, angiotensin II, and serotonin receptors by binding analysis in cell membrane and intact Leydig cells. Primary culture of Leydig cells and testis explants. Development of antibodies to CYP17 in rabbits. Steroid analysis of incubation media by specific radioimmunoassays and of peptides and cyclic nucleotides. Measurements of steroidogenic enzyme activity CYP17 17α-hydroxylase/17-20 desmolase activity in transfected cells with wild type and mutant cDNA by conversion of steroid precursors of $\Delta 4$ and $\Delta 5$ pathways. Measurement of CYP17 concentrations in Leydig cell

microsomal fractions by specific radioimmunoassay. Assay of 17β-hydroxysteroid dehydrogenase activity in rat Leydig cells. Preparation of mRNA from adult and fetal Leydig cells. Transcriptional assays, Northern blot analysis, dot and slot blots, hybridization of blots with 17α-hydroxylase/17 desmolase cDNA probes. Hybridization histochemistry of fetal cell cultures. Screening of rat testis and ovarian libraries and genomic library, DNA sequencing. Transfection of mammalian cells in continuous culture using DOTAP or lipofectamine. Preparation of rat CYP17 wild type promoter and mutants/luciferase fusion genes incorporated into the genome of a replication defective human adenovirus vector by homologous recombination for infections of primary Leydig cell (expressing) and adrenal cell cultures (non-expressing). DNA sequencing. Site-directed mutagenesis using polymerase chain reaction. 17β-hydroxysteroid dehydrogenase (HSD) activity in intact Leydig cells. Expression cloning of 17β-HSD from Leydig cell expression libraries; also using probes from the HSD type 3 human cDNA sequence.

Major Findings:

A. Gonadal receptors: structure, regulation, and function.

Structure and transcriptional regulation of the rat LH receptor gene: Transcription of the rat luteinizing hormone receptor gene is dependent on Sp1-induced promoter activation from two Sp1 binding domains (Sp1_2 and Sp1_4) within the 173-base pair promoter. Of the two Sp1 binding domains, the canonical GC box (GGGCGG) was determined by mutation to be the binding element for only the Sp1_2 domain. The Sp1_4, domain is configured as adjacent non-canonical (GGG GTG GGG) and canonical (GGGCGG) Sp1 elements with a two-nucleotide overlap. The Sp1 binding element within the SP1_4 was identified by mutation and immunological/competition studies as the 5'-GGG GTG GGG (domain b) that conforms to a Zif-268 like three zinc finger binding domain, rather than the canonical 3' Sp1_4 GC box (GGGCGG). The guanines in the third trinucleotide (GGG GTG GGG) were not required for Sp1 binding, although they increased binding affinity. Non-Sp1 protein(s) bind the 3' Sp1_4 GC box, (a domain) and by themselves exhibit transcriptional activity. The two-nucleotide overlap is only essential for binding of the non-Sp1 protein to the a domain, but not for binding of Sp1 to the **b** domain. Therefore, competition or simultaneous binding of the two factors are feasible. The LHR Sp1 binding element, GGG GTG GGG, in which the C of the Sp1 consensus element GGG GCG GGG is replaced by a T, potentially conforms to the Zif-268 DNA binding pattern since the zinc fingers contact the guanines of the GCG trinucleotide, and not the central cytosine. Sp1 does not bind to the GC box in domain a even in the absence of the non-canonical Sp1 binding element, and this may be attributed to a higher affinity for the non-Sp1 proteins. The only nucleotide difference between the Sp1 binding element of Sp1_2 (GGGGCGGGCAGA), and the non-Sp1 binding element of Sp1_4 a (GGGGCGGAGA) is the 3'-AGA extension of the GC box;therefore, these nucleotides play an important role in the binding of specific non-Sp1 factors.

The non-Sp1 trans-activator that binds to domain a may represent different proteins in the non-expressing and expressing cell types. In the presence of CHO nuclear extract, domain a forms three functional protein/DNA complexes, whereas in expressing MLTC nuclear extract, domain a forms only one functional complex. In addition, the MLTC a domain trans-activator is only functional when the **b** element is mutated, whereas the CHO trans-activator is constitutively functional. The Sp1 protein was distinguished from GC binding non-Sp1 protein with immunological supershifts of DNA/protein complexes, using an antibody which binds to regions other than the DNA binding domain. From the several non-Sp1 transcriptional factors that have been reported to bind GC boxes, the BTEB2 protein found predominantly in placenta and testis is a possible candidate (59% similarity with Sp1 in the binding domain, but not similarly in the activation domain).

Both expressing and non-expressing cells exhibit significant levels of p174 transcriptional activation, the presence or absence of the a-binding protein may be linked to regulation of promoter activity by inhibitory upstream domains or the activating downstream M1 domain. The MLTC-specific M1 protein by itself can activate $Sp1_4$-induced LHR gene transcription in the intact p174 promoter, and mutation of the $Sp1_4$ a domain does not further change this promoter activity. However, in the non-expressing cell, the a domain in the p174 promoter can functionally substitute for the activating M1 domain. In these cells simultaneous mutation of both the **a** and M1 subdomains is necessary to effectively inhibit $Sp1_4$-induced p174 LHR promoter activity and removal of these trans-factors from the a and M1 subdomain in the non-expressing cell may play a role in the permanent silencing of gene expression.

Functional glycosylation sites in the LH receptor. The LH is a glycoprotein present in the cell membrane of gonadal cells, with six potential N-linked glycosylation sites and N-linked carbohydrate (CHO) chains of the complex type. In previous studies we have shown that mutation of only three of the six Asn of the LHR putative glycosylation sites (Asn-77, Asn-152, and Asn-173) reduced hormone binding activity, and this could not be attributed to changes in LHR transport to the cell surface. In recent studies we have explored the importance of post-translational glycosylation on LHR binding activity. For this work we have evaluated both the membrane-bound LHR holoreceptor (form A) (S) and its high-affinity hormone-binding splice variant, form B, which lacks the transmembrane and cytoplasmic domains. These receptors and their mutated forms were overexpressed in *Baculovirus*-infected insect cells and expressed in the mammalian COS-1 cells. Overexpression of the holoreceptor was achieved in insect cells at levels of 130-fold greater than that observed in transfected COS cells. The extracellular domain of the receptor was also overexpressed in baculovirus-infected cells at levels of 110 pmol/mg protein. Both overexpressed receptors forms are glycosylated (high mannose) and exhibit high-affinity hormone binding. This procedure is applicable to large-scale preparation of the receptor for use in structural analysis. The receptor binding domain expressed in insect cells was found to be a valuable reagent for use in studies of functional glycosylation sites as a prototype of the high affinity hormone binding domain of the holoreceptor. Functional carbohydrate chains required for hormone binding activity were localized to glycosylation sites at Asn-173 (essential) and Asn-152 (important), and each high mannose chain contributed about 2 kDa to the molecular mass.

Mutation of either Asn-173 or Thr-175 within the glycosylation consensus sequences Asn-Xsa-Thr in the form A or form B LHR proteins abolished binding activity. This loss of activity could not be attributed to deviations in transport to the cell surface, since the same effect was noted in soluble fractions prepared from COS1 cells. Similarly, in the insect cell, the detergent-extracted form A and form B LHR mutants Asn-178 → Gln and Thr-175 → Ala exhibited no detectable hormone binding activity. Radioimmunoassay analysis of expressed wild-type and mutant LHR protein demonstrated that protein levels of the Asn-173 → Gln and Thr-175 → Ala. mutants were not significantly different from wild-type. Thus, loss of binding activity of the mutant LHRs can be solely attributed to defects in hormone binding rather than reduced expression of the receptor. Mutation of the Asn-152 in the form B LHR, expressed from insect cells, resulted in a total loss of hormone binding activity but mutation of Ser-154 to Ala exhibited only a 60% reduction in hormone binding activity. These results were verified in ligand blots where the Asn-152 → Gln mutant exhibited no hCG binding whereas, the Ser-154 → Ala mutant exhibited reduced but visible binding. The reduction of S154A binding activity is due to an increase in the number of inactive nonbinding receptors rather than a change in binding affinity (not shown) or expression. The discrepancy between the total loss of activity by Asn-152 → Gln and the 60% reduction in hormone binding activity by Ser-154 → Ala may represent a negative effect caused by the introduction of Gln, or more likely, and enhancement in activity caused by the substitution of Ser-154 for Ala. Asn-77 was found not to be glycosylated but its mutation to Gln reduced hormone binding by 50%; reflecting its location in a crucial

region of the binding domain (within a leucine zipper motif).

Immunoblot analysis of the double mutant Asn-152/Asn-173 in comparison with the deglycosylated N-glycanase-treated LHR indicated that additional carbohydrate chains at other glycosylation positions (269, 277, or 291) are present on the LHR. The double mutant exhibited an apparent molecular mass of 2 kDa greater than the enzyme deglycosylated (N-glycanase) LHR or the receptor erived from tunicamycin treated cells. Since in our previous studies we have demonstrated that individual mutation of each of the three remaining glycosylation positions at 269, 277, and 291 produce high affinity hormone binding receptor that was capable of transport to the surface of COS1 cells, the putative carbohydrate chains at these positions are either not involved in hormone binding or can be substituted by carbohydrate chains at other glycosylation positions. Our studies demonstrate that high mannose carbohydrate chains are sufficient for the formation of the high affinity hormone binding domain of the form B LHR expressed from insect cells. This suggested that either carbohydrate residues common to both the high mannose and complex chains are central to the formation of the hormone binding domain, or that carbohydrate function occurs in the mammalian cell prior to processing of the high mannose chains to the complex form, as has been reported with the mannose 6-phosphate receptor. Furthermore, the high mannose or complex carbohydrates of the mature LHR are equally capable of refolding the receptor protein.

Recent experiments have focused on the proximal N-acetylglucosamine linked to Asn that is present in both complex and high mannose chains and is retained on the Endo H- or Endo F-treated LHR but not the N-glycanase-treated LHR. This carbonydrate residue was found to be essential for the renaturation of the high affinity hormone binding receptor following electrophoresis on an SDS-denaturing gel (refolding of the mature receptor), and may participate in the folding of the nascent receptor.

Multiple and tissue-specific promoter control of prolactin receptor expression. Prolactin receptors (PRLR) are widely expressed and multiple mRNA transcripts encoding PRLRs are present in prolactin target tissues. Multiple PRLR mRNA species were identified in several tissues, corresponded to the long and short form of the receptors. In the rat ovary, a 9.7 kb mRNA species was identified as coding for the long form of the receptor and was the most abundant transcript, while the 2.1 kb and 1.8 kb species encoding the short form of the receptor were less abundant. In the rat liver, the 1.8 kb mRNA coding for the short form receptor was the predominant species. The diverse functions of prolactin and the wide distribution of its receptors suggested that expression of the PRLR is subject to complex regulation in different target tissues. The diverse actions of prolactin could be manifested by expression of different receptor forms and signal transduction pathways, and also by differential control of PRLR gene transcription in individual target tissues. To investigate the mechanism by which the transcription of the PRLR gene is controlled, we isolated its 5' flanking regions and identified multiple and tissue specific utilization of promoters in the gonads and in the liver. Analyses of the 5' UTRs of PRLR mRNAs expressed in gonadal and non-gonadal tissues, and their genomic organization, revealed three alternate first exons designated as EI$_1$, EI$_2$, EI$_3$. Each of these exons is alternatively spliced to a common exon (exon 2, -115 to -56 nt) that precedes the third exon containing the translation initiation codon. Alternate utilization of exons EI$_1$, EI$_2$ and EI$_3$, as well as alternative splicing of exon 2, generate multiple 5' UTRs in PRLR transcripts. Three functional PRLR gene promoters (PI, PII, and PIII) that initiate the transcription of EI$_1$, EI$_2$ and EI$_3$, respectively were identified. A major transcription initiation site at -549 was determined for EI$_1$ in the ovary by primer extension analysis, conforming to the 5' end derived from the 5' RACE PCR product. Multiple transcription initiation sites at -405, -461 and -506 were identified for EI$_2$ in the liver by both primer extension and S1 nuclease protection analyses. Transcription initiation at -340 and -351 for EI$_3$ were identified in the ovary and in the liver by primer extension analysis, consistent with the 5' ends derived from 5' ends derived from 5' RACE PCR analysis.

Analyses of the three promoter sequences (PI, PII and PIII) have revealed consensus sequences for several transcription factors, which may be important for the basal as well as hormonally regulated promoter activities. Although no canonical TATAA element was observed within an expected distance from the transcription initiation sites, TATA-like sequences (AATAA) were found at -559 in PI, at -408 and -478/-474 in PII. In addition, Sp1 sequences were observed in PI (-449) and PIII (-262, -273. Also Ap1 and Ap2 sites along with several other consensus elements were observed in these promoter regions. Tissue-specific expression of the PRLR mRNA 5' UTRs was revealed by Northern hybridization of PRLR mRNA and Southern hybridization of 5' RACE PCR products using 5' end sequence probes. Northern blots showed that EI_1 was expressed only in the ovary. However, a very low level of EI_1 was detected in Leydig cells, but not in the liver by 5' RACE PCR analyses. EI_2 was exclusively expressed in the liver and is the major form in this tissue. EI_3 is expressed in the three tissues, as the predominant form in Leydig cells, and as minor forms in the ovary and liver.

In addition, it was observed in the ovary that the difference between the 2.1 kb and 1.8 kb mRNA species corresponding to the short form of the receptor (with short cytoplasmic domain) could be accounted for by the presence in the 5' UTR of EI_1 (442 nt) and EI_3 (236 nt), respectively. However, this 5' UTR difference was not resolvable for the 9.7 kb species which encodes the long form of the receptor (with extended cytoplasmic domain). Since both EI_1 and EI_3 sequences were associated with PRLR mRNAs of both receptor forms, it is suggested that the generation of the long and short form of the receptor is independent of promoter specificity, and may result from a postranscriptionally regulated process. In the Leydig cell, only the EI_3 mRNA species was detected on Northern blots with major bands at 9.7 kb and 1.8 kb. In the liver both EI_2 and EI_3 containing species were resolved as one broad 1.8 kb band due to the small size difference between EI_2 (290-390 nt, see below) and EI_3 (236 nt).

Heterogeneity of PRLR mRNA species can arise from differential transcription initiation, alternative splicing of the coding region and 3' alternative polyadenylation. Although the coding region of the long and short forms of the receptor partially accounted for the size difference between the mRNA transcripts encoding the two receptor forms, it is evident from present data that differential transcriptional initiation also contributes to the mRNA heterogeneity (2.1 kb and 1.8 kb). In addition, the large size of the 9.7 kb species, which was more highly expressed in the ovary than in the testis can be accounted for by a long stretch (>7 kb) of 3' UTR [based on the sizes of the 9.7 kb species, which was more highly expressed in the ovary than in the testis, can be accounted for by a long stretch (<7 kb) of 3' UTR [based on the sizes of the coding region (1.8 kb) and the 5' UTRs (0.35 kb and 0.55 kb)].

The finding of three unique 5' end exons in the 5' UTR, which do not appear individually to associate with specific receptor forms, raises an intriguing question as to whether the difference present in the PRLR mRNA 5' UTR may play roles in regulation of the PRLR gene expression post-transcriptionally. It has been shown that the 5' UTR was associated with the mRNA stability as well as the translatability in other genes. Furthermore, the deletion of partial or entire exon 2 in some of the mRNA forms further diversifies the PRLR 5' UTR. Interestingly, the sequence deleted at -93 to -60 can potentially form a stem loop structure and therefore its presence or absence may be significant in regulating the PRLR mRNA stability and/or translatability.

B. The androgen pathway: Hormonal regulation of testicular function.

CYP17 structure and function. Cytochrome P450-17α (CYP17), the key enzyme for the synthesis of androgen and glucocorticoids, catalyses two consecutive oxidation reactions in the $\Delta 5$ and $\Delta 4$ steroid metabolism pathways to produce the 17α-hydroxylated intermediate and androstene products (androgens).

Characterization of the rat CYP17 has revealed that the conserved domain between positions 343 and 363 carries charged aminoacids that contribute to lyase and hydroxylase activities. Arg 346 is esential for both Δ4 and Δ5 lysase activity and Arg 363 is important in the hydroxylase reaction. The substitution of Ile for Phe 343 abolishes the enhacement of Δ4- of over Δ5- lyase activity catalyzed by CYP17, and this substitution is a contributing factor to the absence of Δ4-lyase activity catalyzed by human/bovine CYP17. These findings have emphasized the physical separation of hydroxylase from lyase activity as well as the importance of domain 343-348 for the lyase activity of CYP17. Pulse-chase experiments demonstrated that the substitution of Ala for Glu358 and Arg361 markedly decreases the half-life of CYP17 (from >10 hrs to 1.8 hrs). A homology model of the rat CYP17 was derived from the α/βF supersecondary structural element of the $3\alpha/20\beta$ hydroxysteroid dehydrogenase (HSD) of *Streptomyces hydrogenans*, which constitutes a major segment of the C19 steroid binding cavity. A significant sequence similarity with the CYP17 domain of relevance for activity of the enzyme (334-368), with identities at the essential amino acids Arg346, Arg361, and Arg363, exists in the structural domain of the HSD steroid binding cavity resolved to 2.6 Å by X ray crystallography. This includes sequences between the two domains known to be important for C19 binding. The residues located at folding junctions in the model steroid binding domain [Glu358 and Arg361 at the inner lower core of the pocket, and Tyr431 (at the apex of the HSD substrate binding cavity)] are individually required for correct folding of the active site cavity and their substitution results in an inactive and unstable polypeptide. Genomic analysis of the rat CYP17 gene reveals that this domain is contained in exon 6, and a correlation exists between the length of exon 6 and the boundaries of the HSD supersecondary element. Thus, exon 6 of the rat CYP17 is essential for enzyme activity and may be structurally related to the NAD-linked prokaryote α/βF supersecondary element.

GHRH immunolocalization and actions. We have demonstrated that GHRH is a potent amplifier of FSH-stimulated cAMP production, and that GHRH's stimulation of cAMP generation was most evident in pubertal cells. GHRH also stimulates basal cAMP production in adult and pubertal Sertoli cells and increases intracellular cAMP formation, further indicating that its action on testicular target cells, i.e. Leydig and Sertoli cells, is exerted through the activation of adenylate cyclase. GHRH-like immunoreactivity was localized in interstitual cells, where it exhibited a developmental pattern of expression with the appearance of GHRH-stainable cells during neonatal life (day 4) and a maximal incidence of immunoreactive cells in adult life. Within the seminiferous tubule, GHRH-like immunostaining showed stage-specific localization to the acrosomal region of early to intermediate spermatids (from stages III-IV of the seminiferous epithelium cycle), and no immunoreactivity was found in the late spermatids or mature sperm. These results add a new dimension to the actions and localization of GHRH in the testis.

Interestingly, basal and FSH-dependent tubular cAMP production is much higher at stages II-VI than in stage VII-XII of the spermatogenic cycle, possibly reflecting increased needs of FSH-dependent specialized Sertoli cell functions by germ cells associated with those stages. Thus, as GHRH potently amplifies FSH-stimulated cAMP production, it is possible that this peptide may be stage dependently produced in the testis as a response to local requirement for increased Sertoli cell function. GHRH derived from these cellular elements of the testis could behave as local agonist and/or a potent synergistic factor of FSH action on Sertoli cell functions. In fetal and early post-natal life, FSH is critical for the proliferation of Sertoli cells to the final cell number reached before the onset of meiosis, which is directly related to the ultimate spermatogenic capability. In other epithelial cells (i.e. keratinocytes), it has been shown that VIP acts as a mitogenic factor by stimulating adenylate cyclase via specific VIP receptors. As it is likely that GHRH acts on Sertoli cells, as in Leydig cells, through VIP/GHRH receptors, the peptide present in neonatal Leydig cells may be a potential proliferative agent for the Sertoli cell. It is

possible that GHRH could exert intrinsic functions in the early and intermediate spermatids and contribute to stage-specific cytoskeletal changes together with another protein(s) (i.e. β-chimaerin), which is known to be maximally expressed in germ cells during acrosomal assembly.

Significance to Biomedical Research and the Program of the Institute:

A. Gonadal receptors: structure, regulation, and function. Characterization of LH and prolactin receptors and their relationship to membrane coupling systems will assist in understanding the basic biochemical mechanisms which control the reproductive functions of the testis and ovary. Also, further elucidation of the receptor-cyclase interaction will be of general value in understanding the mechanisms of action of protein hormones.

The recently elucidation of functional domains within the promoter and upstream regions of the LH receptor will permit the complete characterization of the transcriptional mechanism of this atypical promoter, their regulation and the elucidation of processes involved in developmental and tissue-specific expression of the receptor, and its up-and down-regulation at different stages of gonadal development. Analysis of the domains involved in ligand interaction should provide the basis for the development of receptor antagonists. This could also identify critical regions susceptible to mutational inactivation in certain forms of primary hypogonadism. In addition these studies contribute with basic information about mode of transcriptional and regulated activity in tataless genes.

B. The androgen pathway: hormonal regulation of testicular function.

The hormonal control of Leydig cell function and androgen production is an essential component of male sexual development and reproductive capacity. In addition to its significance to the programs of the Endocrinology and Reproduction Research, this project has the potential to provide knowledge about the general actions of peptide and protein hormones upon target cells in tissues other than the gonads.

The findings on the autocrine/paracrine actions of 5HT/CRF/opiates are not only relevant to basic aspects of the local physiological control of testicular function but also provide new insights into the pathogenesis of stress-related disorders of reproductive function, idiopathic infertility and disorders arising from drug addiction and antihypertensive therapy. It should be possible to design therapeutic strategies addressing the two primary alterations that may result from dysfunction of this complex hormonal regulatory system, CRF's direct inhibitory effect on steroidogenesis and its stimulation of opiatergic tone. Both of these actions could lead to disorders of androgen production and alteration of tubule function responsible for some forms of infertility in men. 5HT, the immediate stimulus of testicular CRF secretion, is released centrally and peripherally during stress, and is locally increased in pathological disturbances of testicular function (i.e., orchitis, varicocele). These findings defining the primary participation of 5HT in the secretion of CRF by the Leydig cells provide an important avenue for novel therapeutic approaches for male infertility through the development of antagonist therapy. In studies of CYP17, the physical separation of hydroxylase from lyase activity, and the importance of domain 343-348 for lyase activity in this enzyme, are relevant to the development of inhibitors of androgen formation for treatment of patients with prostate cancer, and for elucidation of the basis of certain forms of male pseudohermaphroditism.

Proposed Course:

A. Gonadal receptors: structure, regulation, and function.

1. Functional analysis of promoter regions of the rat LH receptor gene. We will proceed with further elucidation of specific regions of the LH receptor gene that are responsible for transcriptional initiation and its regulation (including regions relevant to constitutive, hormone-, 2nd messenger-, and growth factor-dependent inducible transcription). Investigation of differences in promoter activity that might account for differential expression of the LH receptor in the ovary and Leydig cell during development. Investigation of transacting factors that are involved in the LH receptor gene regulation. Identification of cis-regulatory elements associated with the rat LH receptor gene which are relevant and for up and down regulation of receptors.
We will extent studies on the characterization of the M1 protein and proceed with analysis of M1 clones recently obtained by expression cloning of a Leydig cell library. After sequencing these clones we will characterize the protein. This will include the identification of functional domains and the sites of interaction with the M1 DNA element and direct or indirect interactions with relevant protein(s) such as SP1 or other proteins that may be involved in basal and activated transcription. In a similar fashion the proteins interacting with SP1 at the $SP1_2$ domain of the rat promoter region will be investigated.

We will initiate studies on the functional characterization of the human LH receptor gene in a similar fashion as was previously performed for the rat receptor. Several length constructs will be expressed in a number of human cell lines. Recent studies have indicated that the LH receptor is expressed in a number of extragonadal human tissues, therefore an initial screening of current human cell lines will be performed prior to adopting a cell line that would be suitable for our studies. Following identification of the promoter domain which presumably resides in areas close to the initiation of transcription site we will mutate the putative 5' atypical TATA boxes and map the regions of transcriptional control.

2. Investigation of regions and specific amino acids of the extracellular domain required for high-affinity binding, for hormonal specificity, and for signal transduction, by means of deletions, construction of chimeras, and mutations. For further characterization of the binding domain, experiments will include deletions and/of additional selective mutagenesis of Cys residues to gain insights on the participation of disulfide bonds (possible assignment) in the structure of the binding pocket. In particular, emphasis will be given to unique LHR Cys residues 109 and 134. Also leucine repeats which are unique for the LHR will be mutated. These structures may contribute to the binding pocket as well as to the specificity of the hormonal signal. For signal transduction, changes will include mutations of the Cys residues within the transmembrane domain as well as point mutations of transmembrane V and VI and of the third intracellular loop and of the N- or C-region of this loop as well as selected areas of the cytoplasmic domain and mutation of individual amino acids within this region. In addition potential regions of interaction between the extracellular binding domain (empty receptor or following occupancy with the transmembrane domain) and the transmembrane domain. Potential phosphorylation sites will be mutated individually.

3. Further elucidation of the role of carbohydrate residues of the LH receptor in the binding of hormone and their participation on the folding and transit of the receptors to the plasma membrane. Specifically the role of N-acetylglucosamine (linked to Asp in the CHO chain) will be investigated. We will attempt to determine the relevant residue(s) within the receptor that interact with this sugar moiety and participate in the folding reaction induced by the sugar residue. Within the established requirement of the sugar residue for folding and or conformational stability of the binding site we will determine whether a

putative fucose attached to the sugar residue participate in the folding of the nascent receptor and conformational stability of the mature receptor.

4. Overexpression of LH receptor forms using a recombinant baculovirus expression system. Large scale production of wild type functional and mutant receptor form B with a range of biological activities and subsequent purification will be obtained for studies on the structure/function aspects of the receptor, for analysis of structural requirements for hormonal binding, and ultimately for analyzing the conformation of the binding pocket.

5. Further characterization of mRNA forms of LH and lactogen receptor. Determination of the nuclear rate of transcription of the LH and prolactin receptor during ovarian and testicular homologous (LH receptor) and heterologous (prolactin receptor) by trophic hormones and 2nd messengers. *In vitro* translation of different mRNA forms or LH and prolactin receptors and identification of products by immunoprecipitation with a battery antibodies to receptor peptides.

6. Further definition of prolactin receptor promoter domains/ tissue specificity in the rat. Assuming that multiplicity of promoter/and tissue specificity is similar for the mouse as was found in the rat we will proceed with selective knock out of the individual promoter that selectively direct the expression of the receptor in testis and ovary. We will proceed with studies on the transcriptional regulation of LH and prolactin receptors by homologous and heterologous hormones.

B. The androgen pathway: hormonal regulation of testicular function.

1. We will further extend the analysis of mechanisms responsible for induction of early and late steroidogenic lesions. These will include studies on the regulation of gene expression of Leydig cell cytochrome P-450 17α-hydroxylase, 17-20 desmolase by hCG and estradiol, and nuclear transcription assays. We will determine structural requirements of the P-450$_{17\alpha}$ for enzymatic activities of 17α-hydroxylase/17-20 desmolase, and will isolate the 5' regulatory gene sequences for investigation of regulation of gene function. We will study direct actions of estradiol, androgen or other modulators on P450$_{17\alpha}$ gene regulation. The expression of enzymatic activities of constructs with P450$_{17\alpha}$ cDNA inserts with deletions of the 1st and/or 2nd transmembrane regions, and in some cases containing a signal peptide of secretory preproteins, will be explored in COS-1 cells. Also, we will carry out expression of P450$_{17\alpha}$ cDNA with selective mutations of regions of the putative binding site to explore further the individual domains of this bifunctional enzyme.

2. Regulation of the last step of the steroid biosynthetic pathway is under investigation. Preliminary studies indicate that activation of the enzyme requires ATP and the enzymatic activity is dependent of adequate entry of glucose into the cells. The rat HSD will be cloned by expression cloning, and or hybridization using oligonucleotide probes from the recently cloned human HSD type 3 isoform. The enzyme will be overexpressed and regulation of its activity will be investigated. Also, HSD cDNA constructs wild type and mutants, will be coexpressed in COS1 cells with CYP17 and the activity of the enzyme will be determined. The role of phosphorylation on the activity of the enzyme will be initially investigated putative phosphorylation sites will be mutated and the activity of the enzyme will be determined.

3. The mechanisms of LH-induced receptor regulation and steroidogenic lesions will be analyzed in cultured Leydig cells. More detailed analysis of the appearance and development of receptors and responses through fetal life, and the influence of homologous and heterologous hormones such as

prolactin and FSH and GnRH in culture, will be performed. Further investigations of functional compartmentalization of cAMP will be continued with localization and identification of protein phosphorylation substrates during the action of LH, choleragen and forskolin, along with other stimulatory and inhibitory hormones and substances. This is to be correlated with adenylate and guanylate cyclase activities, cAMP production, and steroidogenesis in fetal and adult Leydig cells.

4. Communications between tubule cells and Leydig cells, and myoid and mesenchymal cells with Leydig cells, will also be studied. The receptors and modulatory actions of a number of peptide hormones of known stimulatory and inhibitory actions in other tissues will be analyzed in Leydig, Sertoli, and germinal cells in attempts to understand homologous regulatory mechanisms within cell populations. We will investigate the nature of the serotonin signal transduction mechanism and membrane coupling functions, and will further dissect the mechanism of the gonadotropin signaling pathway affected by CRF. The dual role of protein kinase C as a mediator of CRF, and presumably of serotonin action in Leydig cells, will also be evaluated.

Publications:

Buczko, E., Koh, Y.C., Miyagawa, Y., and Dufau, M.L. The rat 17α-hydroxylase-17,20-desmolase (CYP17) active site: computerized homology modeling and site directed mutagenesis, J. Steroid Biochem. Mol. Biol. 52:209-218, 1995.

Dufau, M.L. The luteinizing hormone receptor. Current Opinion in Endocrinology and Diabetes 1995;2:365-374.

Dufau, M.L., Tsai-Morris, C-H., Hu, Z.Z., and Buczko, E. Structure and regulation of the luteinizing hormone receptor gene, J. Steroid Biochem Molec. Biol. 53:286-291, 1995.

Dufau, M.L., Tsai-Morris, C.-H., Hu, Z.Z., Geng, Y., Xie, X., Zhuang, L., and Buczko, E. The luteinizing hormone receptor. In Dufau, M.L., Fabbri, A., Isidori, A., eds. Frontiers in Endocrinology: Cell and Molecular Biology of the Testis. Ares-Serono Symposia. Vol. 5:51-60, Ares-Serono Symposia Publications, Rome, Italy, 1994.

Fabbri, A, Ciampani, T., Aversa, A., Dufau, M.L., and Isidori, A. Hypothalamic releasing hormones in the testis: role of corticotropin releasing factor and growth hormone releasing hormone. In Dufau, M.L., Fabbri, A., Isidori, A., eds. Frontiers in Endocrinology: Cell and Molecular Biology of the Testis. Ares-Serono Symposia. Vol. 5:155-164, Ares-Serono Symposia Publications, Rome, Italy, 1994.

Fabbri, A, Ciocca, D., Ciampani, T., Wang J. and Dufau, M.L. Presence of growth hormone releasing hormone (GHRH) intesticular intestinal and germ cells potential paracrine modulation of FSH action on Sertoli cell function. Endocrinology 136:2303-2308, 1995.

Tomic, M., Dufau, M.L., Catt, K.J. and Stojikvocic, S. Calcium signaling in single rat Leydig cells, Endocrinology. 136:3422-3429, 1995.

Tsai-Morris, C-H., Geng, Y., Xie, X-Z., Buczko, E., and Dufau, M.L. Transcriptional protein binding domains governing basal expression of the rat luteinizing hormone receptor gene. J. Biol. Chem. 1994;269:15868-15875.

Tsai-Morris C-H, Geng-Y, Buczko, E. and Dufau, M.L. Characterization of diverse functional elements in the upstream Sp1 domain of the rat luteinzing hormone receptor gene promoter (1995) J. Biol. Chem. 1995;270:7487-7494.

Veldhuis, J.D., and Dufau, M.L. Dynamics and pathophysiology of biologically active LH secretion in man. In Dufau, M.L., Fabbri, A., Isidori, A., eds. Frontiers in Endocrinology: Cell and Molecular Biology of the Testis. Ares-Serono Symposia. Vol. 5:89-98, Ares-Serono Symposia Publications, Rome, Italy, 1994.

Veldhuis, J.D., and Dufau, M.L. Differential responses of biologically active LH secretion in older versus young men to interruption of androgen negative feedback. J. Clin. Endocrinol. Metab. 1995;79:1763-1770.

Zhang, R., Cai, H., Fatima, N. Buczko, E. and Dufau, M.L. Functional glycosylation sites of the rat luteinizing hormone receptor required for hormone binding. J. Biol. Chem. 1995, 270:21722-21728, 1995.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00184-17 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Pituitary Hormone Secretion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K.J. Catt	Head, SHR	ERRB, NICHD
Other:	K. Arora	Sr. Staff Fellow	ERRB, NICHD
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LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structural and signaling properties of receptors for gonadotropin-releasing hormone (GnRH), and their role in neuropeptide release and gonadotropin secretion, were investigated in hypothalamic and pituitary cells. GnRH receptors were previously cloned and found to be expressed not only in pituitary gonadotrophs but also in the hypothalamus and in immortalized GnRH neurons (GT1 cells). In both perifused GT1 cells and hypothalamic neurons, GnRH agonists influence the normal pulsatile secretion of GnRH by their action on GnRH receptors. GT1 cells also express receptor channels for glutamate (NMDA and AMPA/kainate receptors) and for gamma-amino-butyric acid (GABA$_A$ receptors). The combined effects of glutamate and GABA on cytoplasmic calcium responses were additive and were sensitive to nifedipine. However, their combined effects on GnRH release were more than additive, consistent with non-linear coupling of calcium to exocytosis. In pituitary gonadotrophs the effects of progesterone on GnRH-stimulated LH release were accompanied by significant changes in the calcium signaling responses to agonist stimulation, with stimulatory or inhibitory actions according to the duration of progesterone treatment. Also, GnRH was found to activate both phospholipase C and D in a sequential and synchronized manner via activation of protein kinase C. Studies on the control of exocytosis in permeabilized pituitary gonadotrophs showed that while elevated cytoplasmic calcium per se can promote LH release, concomitant activation of protein kinase C is necessary to support hormone secretion at the physiological calcium levels that prevail in agonist-stimulated intact cells. Thus, sensitization of the calcium-dependent secretory mechanism by protein kinase C may be an important step in the GnRH-induced release of LH from pituitary gonadotrophs. The rat GnRH receptor gene was found to consist of three exons that encode the receptor protein, and to span about 20 kb of DNA. The reading frame is interrupted by two exons of 12 and 2.5 kb in the 4th and between the 5th and 6th transmembrane domains, respectively, and the promoter region of the gene contains a putative TATA box and several potential regulatory elements. In structure-function studies of the GnRH receptor, a conserved hydrophobic residue (Leu^{147}) in the second intracellular loop was found to be essential for receptor signaling and endocytosis. In contrast, restoration of tyrosine in place of the unusual serine residue present in the DRS motif that replaces the conserved DRY sequence present in most G protein-coupled receptors did not affect receptor signaling but increased agonist binding affinity and receptor internalization.

Project Description

Objectives:

To clarify the mechanisms of action of hypothalamic neurohormones and other regulatory factors that control secretion in gonadotropin-releasing hormone (GnRH) neurons and anterior pituitary cells. In particular, to characterize the receptors, signal transduction pathways, and cellular processes involved in the neuronal control of GnRH biosynthesis and secretion, and the effects of GnRH on gonadotropin release. This includes studies on the GnRH-producing neurons of the hypothalamus, immortalized GnRH-secreting neurons, cultured pituitary cells, and αT3-1 gonadotroph cell lines to elucidate the receptor-mediated pathways that control the functions of these cell types.

Methods Employed:

Dispersion and culture of hypothalamic and pituitary cells, and αT3-1 and GT1-7 cell lines. Radioligand assays, solubilization, and photoaffinity labeling of GnRH receptors in pituitary cells and membrane-rich fractions. Column perfusion of cultured pituitary cells and hypothalamic neurons. Radioimmunoassay of cyclic nucleotides, gonadotropins, prolactin, and other hormones in pituitary cells and culture medium. Fractionations of pituitary cell suspensions by density gradient and centrifugal elutriation methods. Measurements of inositol phospholipid turnover and arachidonic acid metabolites in gonadotrophs labeled with radioactive precursors. Assay for diacylglycerol, and of protein kinase C and its distribution in ligand-stimulated cells. Measurements of cytoplasmic calcium concentrations in single cells by photometry and imaging techniques, and in suspension by spectrofluorimetry. Cloning and mutational analysis of GnRH receptors; analysis of pathways and transcription factors regulating GnRH receptor gene expression.

Major Findings:

Regulation of $[Ca^{2+}]_i$ signaling and GnRH release by neurotransmitters. Both γ-aminobutyric acid (GABA) and glutamate have been postulated to modulate gonadotropin-releasing hormone (GnRH) secretion from GnRH neurons as part of their widespread role in hypothalamic function. This proposal is supported by both anatomical and physiological evidence. In rats, GABA-containing neurons make extensive synaptic contact with GnRH neurons in the preoptic area (axo-somatic) and at the level of the median eminence (axo-axonic). In monkey, glutamate-containing neurons form synapses with GnRH neurons in the organum vasculorum of the lamina terminals, the medial portion of the ventral hypothalamic tract, and the arcuate nucleus (axo-dendritic). GABA and glutamate may act jointly to modulate GnRH secretion. However, it not at present clear whether GABA and glutamate act directly on GnRH neurons to increase GnRH, and whether synchronous activation of GABA and glutamate receptors is required for a maximal response to the combination of both agonists. Moreover, the mechanism of the secretory response to combined stimulation by GABA and glutamate has not been defined.

We addressed these issues in studies on the GT1-7 cell line of immortalized GnRH neurons. $[Ca^{2+}]_i$ was increased by GABA and the $GABA_A$ receptor agonist, muscimol, and these responses were inhibited by the $GABA_A$ receptor antagonist, bicuculline. In contrast, the $GABA_B$ receptor agonist, baclofen, and the $GABA_B$ receptor antagonist, phaclofen, had no effect on basal and GABA- and glutamate-induced Ca^{2+} levels in GT1-7 neurons. The GABA- and muscimol-induced responses consisted of a spike increase in $[Ca^{2+}]_i$ followed by a decrease to a plateau; both the increase and the

subsequent decrease in $[Ca^{2+}]_i$ depended on agonist concentration. Glutamate, NMDA and kainate also increased $[Ca^{2+}]_i$, but were less effective than GABA. Glutamate-, NMDA-, kainate- and GABA-induced $[Ca^{2+}]_i$ responses were almost abolished in Ca^{2+}-free medium and were markedly attenuated by nifedipine. The calcium response to GABA was unaffected by prior application of glutamate, and vice versa. This additive effect of glutamate on the GABA-induced $[Ca^{2+}]_i$ responses was mimicked by prior and simultaneous application of low (10 mM) KCl. The $[Ca^{2+}]_i$ response to simultaneous application of GABA and glutamate was also equal to the sum of the individual responses, whereas the GnRH secretory response was larger. However, the secretory responses to GABA and glutamate applied individually or together, were markedly attenuated by nifedipine. These results indicate that in GT1-7 neurons the combined actions of GABA and glutamate on $[Ca^{2+}]_i$ are additive of the actions of each transmitter, which are nifedipine-sensitive. However, the combined actions of the two transmitters on GnRH release are more than additive, suggesting the existence of non-linear coupling between $[Ca^{2+}]_i$ and exocytosis in neuroendocrine cells.

GnRH-induced sensitization of calcium-dependent exocytosis in pituitary gonadotrophs. In a wide variety of secretory cells, regulated exocytosis is characterized by the rapid and coordinated release of peptides or proteins from storage granules during agonist stimulation. In endocrine and neuroendocrine cells, cytoplasmic Ca^{2+} is a critical component of the signaling pathway that leads to regulated exocytosis. In addition, several plasma membrane, cytoplasmic, secretory vesicle, and cytoskeletal proteins have been linked to the activation of exocytosis in agonist-stimulated cells. The mechanisms by which these proteins act in conjunction with Ca^{2+} to promote secretion are not clear, but it is probable that several proteins act in concert to translate the Ca^{2+} signal into the secretory response. Certain cytoplasmic proteins that are highly sensitive to changes in intracellular $[Ca^{2+}]$, including calmodulin and protein kinase C isozymes, have received much attention as mediators of exocytosis. At present, the mechanisms by which the coordinate actions of Ca^{2+} and cytoplasmic proteins, including protein kinase C, control the initiation and rate of exocytosis remain unknown. Furthermore, the involvement of protein kinase C in receptor-controlled exocytosis has been questioned in several cell types that are controlled by Ca^{2+}-mobilizing agonists.

We investigated the interactions between Ca^{2+} and protein kinase C during activation of exocytosis in preparations of permeabilized rat pituitary gonadotrophs that respond not only to increases in $[Ca^{2+}]$ but also to agonist stimulation by GnRH. In permeabilized and ATP-primed pituitary cells, increases in ambient $[Ca^{2+}]_i$ stimulated the release of luteinizing hormone (LH) from gonadotrophs with an EC_{50} of 2-3 μM. In contrast, the responses of intact gonadotrophs to agonist stimulation by GnRH were characterized by transient $[Ca^{2+}]_i$ elevations of up to 1.5 μM, followed by a plateau of 300-400 nM. The sensitivity of the exocytotic response of permeabilized cells to $[Ca^{2+}]$ was significantly increased by GnRH, which reduced the EC_{50} for $[Ca^{2+}]$ to the sub-micromolar concentration range. The stimulatory action of GnRH on LH release in permeabilized cells was not a consequence of intracellular Ca^{2+} release, but was associated with increased cytidine diphosphate diacylglycerol production. Activation of protein kinase C by phorbol esters caused a similar increase in the Ca^{2+}-sensitivity of LH release from permeabilized cells, and this effect was not additive to that of GnRH. Furthermore, the GnRH-induced increase in sensitivity of the exocytotic response to Ca^{2+} was attenuated by inhibitors of protein kinase C. These findings indicate that while elevated $[Ca^{2+}]$ per se can promote LH release from permeabilized gonadotrophs, concomitant activation of protein kinase C is necessary to support exocytosis at the physiological $[Ca^{2+}]_i$ levels that prevail in GnRH-stimulated intact cells. Such sensitization of the calcium-dependent secretory mechanism by protein kinase C may be an important step in the agonist-induced release of LH from pituitary gonadotrophs.

Modulation of calcium signaling and gonadotropin release by steroid hormones. Estradiol and progesterone are important ovarian modulators of gonadotropin secretion from the anterior pituitary gland. The positive and negative feedback effects of these steroids include both sensitizing and inhibitory actions on the responsiveness of pituitary gonadotrophs to hypothalamic GnRH. In cultured pituitary cells from female rats, long-term (> 12 h) estradiol treatment leads to enhanced LH and FSH responses to GnRH stimulation. In such estrogen-primed cells, short-term (> 45 min) progesterone treatment exerts an acute facilitatory effect on GnRH-stimulated gonadotropin secretion. Conversely, prolonged exposure (> 16 h) of these cells to progesterone leads to inhibition of LH and FSH secretory responses. Although the effects of gonadal steroids on gonadotropin release are well characterized, the underlying mechanisms of these actions are poorly understood.

To address the specificity of progesterone actions, we have employed several progesterone antagonists. The antiprogestins RU486, ZK 98.299, and ZK 98.734 blocked both the acute facilitatory and the long-term inhibitory action of progesterone. In the absence of progesterone, the antiprogestins per se induced marked inhibitory and stimulatory effects on GnRH-stimulated LH secretion. In brief, short-term treatment of non-oestrogen-primed cells with antiprogestins was either ineffective (ZK compounds) or reduced LH secretion (RU486), while long-term treatment was stimulatory. In oestrogen-primed cells, these compounds exerted purely inhibitory effects on GnRH-induced LH secretion. Thus, antiprogestins are effective antagonists of progesterone actions in the gonadotroph. However, in the absence of progesterone, they exert diverse actions on gonadotropin secretion that can interfere with their antagonistic properties.

Structure-function studies on GnRH receptors. Following our cloning of cDNA encoding the mouse GnRH receptor, the rat, sheep, cow, and human receptors have been cloned and found to share about 85% amino acid homology among species. The coding region of the GnRH receptor contains seven putative transmembrane domains (TM I-VII) as well as many of the conserved residues and sequences typical of other members of the G protein-coupled receptor (GPCR) superfamily. However, the GnRH receptor exhibits several unique features that include (a) the presence of a long and highly basic first intracellular loop; (b) the replacement of Tyr by Ser in the conserved GPCR "signature" motif DRY located at the junction of TM III and the second intracellular loop; and (c) the absence of a cytoplasmic COOH-terminal tail. Another interesting feature of the receptor is that the highly conserved Asp in TM II and Asn in TM VII are reciprocally exchanged. Upon agonist binding, the GnRH receptor stimulates phosphoinositide hydrolysis to diacylglycerol and inositol 1,4,5-trisphosphate, thereby mobilizing Ca^{2+} and activating protein kinase C to initiate a variety of cellular responses. Receptor activation is followed by extensive internalization and down-regulation of the receptor via endocytosis and processing. The molecular mechanism(s) of GnRH receptor activation have yet to be clarified.

Mutagenesis and chimeric studies have suggested that the intracellular regions of the G protein-coupled receptor, in particular the second and third intracellular loops and sometimes the cytoplasmic tail, interact with G proteins and mediate signal transduction. Most of these structural and functional studies have been performed on β-adrenergic and muscarinic receptors, and data on these properties of the peptide hormone receptors are more limited. In addition, recent reports have suggested that the domains/residues involved in G protein activation and receptor internalization processes are multi-site in nature and that some of these regions may either be similar or overlapping. In the muscarinic receptor, a conserved leucine residue in the second intracellular loop has been implicated in both signal transduction and receptor sequestration. This hydrophobic amino acid is also conserved (as Leu[illegible]) in the GnRH receptor and is present at the equivalent position in the 2i loop. In studies on the structure-function relationships of the GnRH receptor, we examined the roles of the conserved Leu[illegible] residue and

the unique Ser^{140} residue, which is Tyr or Trp in most other GPCRs, in agonist-induced signal transduction and receptor internalization. To this end, these residues were replaced with other amino acids by site-directed mutagenesis, and the expressed receptors were analyzed for ligand binding, GnRH-induced inositol phosphate formation and agonist-induced internalization. The replacement of Leu^{147} with either Asp or Ala was found to impair both G protein coupling and receptor internalization. However, replacement of Ser^{140} by Try did not affect the signaling response but increased ligand binding and receptor internalization.

Structure of the rat GnRH receptor gene.

The structure of the rat gonadotropin-releasing hormone receptor gene was determined by analysis of two clones that were isolated by screening a rat testis genomic library with the cDNA for the mouse GnRH receptor. The gene was found to consist of three exons that encode the receptor protein, and to span about 20 kb of DNA. One genomic clone contained the 5'-untranslated region and the first exon, and the other contained the second and third exons. The sizes of the first, second, and third exons are 625, 217, and 1476 nt, respectively. The first intron is at least 12 kb in length and is located between nucleotides 522 and 523 of the cDNA reading frame, in the middle of the fourth transmembrane domain. The second intron is about 2.5 kb and is also located in the reading frame. It is positioned between nucleotides 739 and 740, separating the fifth and sixth transmembrane domains. Thus, the intron-exon structure of the rat receptor gene is similar to those of the mouse and human genes, with intervening sequences located in the coding region at the sites of the fourth transmembrane domain and the third intracellular loop. Genomic blots in combination with cloning and sequencing suggested that a single GnRH receptor gene is present in the rat genome. Primer extension with the DNA polymerase and MMLV (RNAse H-) reverse transcriptase indicated that the transcription start site is located 103 nt upstream of the start codon. A putative TATA box is positioned 126 nt upstream of the start codon and 23 nt in front of the transcription initiation site. The 1.7 kb promoter sequence contains an AP-1 site, CCAAT sequences, and a Pit-1 binding site. In addition, a potential SF-1 binding site is present in the 5'-untranslated region of the receptor sequence. The sequences of the promoter, exons, and introns of the rat GnRH receptor gene are approximately 85% identical with the corresponding regions of the mouse receptor gene.

Significance of Biomedical Research and the Program of the Institute:

The clarification of receptor-mediated mechanisms by which neurotransmitters and other regulators control GnRH release, as well as the effects of GnRH on the development and function of gonadotrophs is essential for understanding of normal and disordered hypothalamo-pituitary functions. The regulation of gonadotropin secretion is of central importance in reproductive endocrinology, and the roles of GnRH, steroid hormones and other factors in this process, and their respective biochemical mechanisms of action, need to be more fully elucidated. The effects of GnRH on reduction of its receptor mRNA levels implicates the participation of an ultra-short feedback effect of GnRH on receptor synthesis that is of potential importance for use in fertility regulation and suppression of pituitary function. The expression of GABA and glutamate receptors in GnRH neurons, and their coupling to calcium signaling and GnRH release, provides a useful model for further investigation of this type of neuronal receptors in the control of neuroendocrine function.

Proposed Course:

Further studies on the signaling and secretory profiles of normal hypothalamic neurons and GT1 immortalized neurons will be pursed in experiments with several peptide agonists and neurotransmitters. Specific attention will be given to the characterization of an intrapituitary GnRH system, as well as to interactions between hypothalamic and pituitary GnRH systems and the kinetic aspects of the receptor activation and desensitization during the integrative actions of these two systems. Also, characterization of the inhibitory factors involved in control of GnRH release, including the possible effects of the pituitary hormones, LH and prolactin, on signaling and secretion by GnRH neurons are planned. The characterization of endothelin receptors in hypothalamic cells, and the role of endothelin as a regulator of GnRH and gonadotropin release, will be further analyzed. The structure-function properties of the GnRH receptor gene and the GnRH receptor will also be further investigated, including mutational analysis to determine the regions involved in ligand coupling to G protein(s) and internalization. Experiments with permeabilized gonadotrophs and αT3-1 cells will be performed to introduce specific antibodies against the intracellular domains of GnRH receptors. Permeabilization studies will also be explored in investigation of the molecular mechanism of exocytosis in gonadotrophs and GnRH neurons, with specific emphasis on the functions of protein kinase C. In this regard, characterization of the isozymes of protein kinase C in pituitary cells, αT3-1 gonadotrophs, and GT1 neurons will be done, as well as the translocation of those enzymes in agonist-stimulated cells. Also, activation of MAP kinase by GnRH, and the coordinate actions of MAP kinase and protein kinase C in cellular functions will be investigated.

Publications:

Cesnjaj M, Zheng L, Catt KJ, Stojilkovic SS. Dependence of stimulus-transcription coupling on phospholipase D in agonist-stimulated pituitary cells. Mol Biol Cell 1995 (in press).

Jobin RM, Tomic M, Zheng L, Stojilkovic SS, Catt KJ. GnRH-induced potentiation of calcium-dependent exocytosis in pituitary gonadotrophs. Endocrinology 1995;136:3398-3405.

Ortmann O, Catt KJ, Schulz KD, Emons G. Modulatory action of progesterone and progesterone antagonists on hypothalamic-pituitary function. Human Reproduction 1994;9:53-62.

Spergel DJ, Krsmanovic LZ, Stojilkovic SS, Catt KJ. L-type Ca^{2+} channels mediate join modulation by GABA and glutamate of [Ca^{2+}]$_i$ and neuropeptide secretion in immortalized GnRH neurons. Neuroendocrinology 1995;61:499-508.

Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995 (in press).

Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995 (in press).

Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of GnRH receptors. Rec Prog Horm Res 1995;50:161-205.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second International Symposium on Fertility Regulation 1995 (in press).

Tomic M, Dufau ML, Catt KJ, Stojilkovic SS. Calcium signaling in single rat Leydig cells. Endocrinology 1995;136:3422-3429.

Zheng L, Paik W-Y, Cesnjaj M, Balla T, Tomic M, Catt KJ, Stojilkovic SS. Effects of the phospholipase-C inhibitor, U73122, on signaling and secretion in pituitary gonadotrophs. Endocrinology 1995;136:1079-1088.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00187-16 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormonal Regulation of Cellular Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Endocrinology and Reproduction Research Branch

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6	6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Covalent modifications of protein by phosphorylation and oxidation are important mechanisms for the modulation of numerous cellular functions. Protein phosphorylation by protein kinase C (PKC) has been linked to the regulation of growth, differentiation, secretion, cellular metabolism, memory, and sensory transduction. Multiple PKC subspecies have been identified by molecular cloning and the various isozymes have been shown to have distinct tissue, cellular, and subcellular distributions and are differentially expressed during development. The functional roles of these kinases in the control of cellular differentiation were tested with N1E115 neuroblastoma cells. PKC-activating phorbol ester counteracts the cAMP-induced neurite outgrowth of these cells by selective translocation and downregulation of PKC alpha and delta, whereas PKC beta and zeta are not greatly affected. PMA stimulates phosphorylation of several proteins including a 95 kDa elongation factor 2, its phosphorylation is attenuated by cAMP. Elucidation of the PKC-regulated events also places emphasis in the characterization of the substrate. A novel 28 kDa PKC/casein kinase II substrate has been identified and its cDNA cloned. This protein is a substrate of casein kinase II in N1E115 neuroblastoma cells. The extent of phosphorylation and protein level are cell cycle-regulated. Another potential PKC substrate, p14.4, has been identified and its cDNA cloned. This protein can also be phosphorylated by p34cdc2 kinase. Neurogranin (Ng) is a calmodulin (CaM)-binding PKC substrate. Its phosphorylation by PKC reduces the affinity for CaM and thus frees CaM for the CaM-dependent enzymes. In addition, oxidation of Ng in air or by peroxide or nitric oxide results in intramolecular disulfide bond formation and reduces the affinity of this protein for CaM. Site-directed mutagenesis of the four Cys residues in Ng has lead to identification of a pair of Cys residues involved in disulfide bond formation. Ng is an excellent acceptor for nitric oxide; the rate of nitrosation of Ng sulfhydryl groups and subsequent disulfide bond formation is even faster than one of the best known reductants, DTT. Modification of Ng by oxidation could be an important signaling mechanism of nitric oxide in the CNS. The genomic structures of the CNS-specific PKC gama and Ng were analyzed for the purpose of defining tissue-specific and development-regulated expression of these two genes. The promoter activity of the Ng gene can be stimulated by phorbol ester or by co-transfection with PKC cDNAs. A 20 kDa protein that binds to the AT-rich regions of the PKC gamma and Ng gene promoters has been identified; phosphorylation of this protein by PKC attenuates its binding to DNA elements containing ATTA, ATAA and AATA motifs.

Project Description

Objectives:

To investigate the hormonal regulation of cellular functions in normal and disordered target tissues. Effort is focused on the delineation of the signal transduction mechanisms in different tissues involving protein kinase C- and redox-regulated cellular functions. Current studies are directed to investigate the role of protein kinase C in mediating the actions of hormones and neurotransmitters; to identify novel protein kinase C substrates in the brain; to determine the role of protein kinase C in secretory responses and modulation of ion channels; to determine the signaling mechanism of nitric oxide in CNS; and to determine the developmental control of protein kinase C and neurogranin gene expressions in neural tissues.

Methods Employed:

This project utilizes a variety of chemical, biochemical, and immunological techniques to study the hormonal regulation of metabolic processes at the molecular and cellular levels. Various enzymes were purified from mammalian tissues and enzyme activities were assayed by spectrophotometric or radiometric methods. Purified enzymes were characterized by their molecular weights as determined by gel electrophoresis, gel filtration, and density gradient centrifugation, by their kinetic properties, and by their substrate specificities. The effects of protein phosphorylation and oxidation were defined by the changes in the activity related parameters and the sites of modification. The phosphorylation sites, defined by the characteristic tryptic peptides, were identified by peptide mapping on thin-layer plate, by isoelectric focusing on polyacrylamide gel, by high-performance liquid chromatography, and by amino acids sequence analysis. Monoclonal antibodies were prepared by hybridoma technology and purified by high-performance liquid chromatography. Monospecific antibody against an antigen was prepared by specific absorption of polyclonal antibodies to nitrocellulose membrane containing previously transferred antigen. Immunological characterization of the antigen was carried out by immunoblotting and by light and electron microscopy. Identification of specific mRNA was carried out by Northern blot analysis using DNA probes obtained by molecular cloning. Characterization of specific gene product was done by transfection of COS cells and followed by biochemical and immunological identification of expressed protein. Recombinant proteins were produced in bacteria. Genomic structure was analyzed by molecular cloning and DNA sequence analysis. Identification of specific DNA-binding protein was carried out by gel-retardation and footprinting analyses.

Major Findings:

Identification of neurogranin as a redox-sensitive calmodulin-binding protein. Neurogranin (Ng), also known as RC3 or BICKS, is a 78-amino acid postsynaptic protein kinase C (PKC) substrate present in the neurons of the adult rodent brain. This protein resembles a presynaptic PKC substrate neuromodulin (also known as GAP-43, F1, or B-50) in its high affinity binding of calmodulin (CaM) at low levels of Ca^{2+}. Both Ng and neuromodulin are phosphorylated by PKC at a single site located within a conserved 18-amino acid sequence region, which also contains the predicted CaM-binding domain. Phosphorylation of these two proteins by PKC weakens their binding affinity for CaM. It has been hypothesized that the PKC-catalyzed phosphorylation of these two proteins frees the CaM for other CaM-dependent enzymes. In the brain, this signal amplification step following the activation of PKC has been linked to the modulation of ion channel and neurotransmitter release and the induction and maintenance

of long-term potentiation. As the expression of Ng increases during development and activation of several PKC isoforms could trigger elevated expression of this gene, this protein may possess other function besides serving as a reservoir for CaM. We have shown that rat brain Ng was readily oxidized in air, by H_2O_2, and nitric oxide donors, DEANO (1,1-diethyl-2-hydroxy-2-nitrosohydrazine) and sodium nitroprusside to generate a large conformational change that could be detected by SDS-polyacrylamide gel electrophoresis without sulfhydryl reagent. The reduced Ng had an apparent M_r = 17k and the oxidized one M_r = 10k; reversible conversion between these two M_r species were seen by treatment of Ng with these oxidants and followed by reduction with sulfhydryl reagent. Both the non-phosphorylated and PKC-phosphorylated Ng were susceptible to the oxidation-induced changes in M_r; modification of all four cysteine residues in Ng with 4-vinylpyridine prevented the oxidation-induced changes. Modification of Ng by DEANO was inhibited by calmodulin (CaM) without Ca^{2+} and in the presence of Ca^{2+}, CaM could not protect Ng from oxidation by DEANO. CaM had no protective effect on DEANO-mediated oxidation of PKC-phosphorylated Ng with or without Ca^{2+}. Oxidation of Ng by the various oxidants resulted in the formation of intramolecular disulfide bond that exhibited lower M_r on SDS-PAGE; this oxidized form displayed lower affinity for CaM than the reduced form as evidenced by CaM-affinity column chromatography. By comparison with the rate of oxidation of Ng by DEANO with other sulfhydryl reagents, Ng ranks as one of the best nitric oxide acceptors among those tested including dithiothreitol, glutathione, and serum albumin. This study suggests that Ng is a likely target of nitric oxide, in addition to PKC, and that modification of Ng by nitric oxide and other oxidants could serve as a signaling mechanism for controlling the level of CaM, which is essential for many Ca^{2+}-regulated cellular processes. In addition, Ng may serve as an anti-oxidant in the neuron to protect from assault by reactive oxygen intermediates following vascular stroke.

Identification of critical cysteine residue in neurogranin responsible for oxidation-induced disulfide bond formation. Ng is readily modified by oxidants such as hydrogen peroxide, nitric oxide, and iodosobenzoic acid to form an intramolecular disulfide bond. The resulting oxidized Ng has a low affinity for calmodulin and becomes a weaker substrate for protein kinase C. Modification of Ng by nitric oxide is apparently mediated by the formation of a nitroso intermediate followed by disulfide bond formation. Ng molecule contains four cysteine residues, $Cys^{3,4,9,51}$, all of which are likely targets of nitric oxide. Constructs containing single, double, and tetra mutants of the four cysteine residues of rat brain Ng were generated for examining the role of individual cysteine residues in forming intramolecular disulfide bond. The mutant proteins were produced in *E. coli* and purified to near homogeneity by acid treatment and HPLC on a C_4 reverse phase column. Mutation of four Cys residues or single mutation at Cys^{51} prevented the oxidant-mediated conversion of the M_r = 17 k to the 10 k species. Single mutation at Cys^3 or Cys^4 had no significant effect and at Cys^9 greatly reduced the disulfide bond formation. These results indicate that Cys^{51} preferentially forms a disulfide bond with Cys^9. In fact, the double mutant C3S-C4S, with Cys^9 intact, formed the oxidized 10 k species equally well as the wild type. The reduced forms of the wild type and all the Cys mutants bound to a CaM-Sepharose affinity column. The CaM-binding affinities of the tetra-Cys and C51G mutants were not affected by oxidants as expected in the absence of conversion of the 17 k to the 10 k species. Oxidation of the wild type and other mutants resulted in loss of binding to the CaM affinity column in proportion to their extent of conversion to the 10 k species. Oxidation of the wild type and those mutants that form M_r = 10k species caused a reduction in the rates of phosphorylation by PKC. This study defines the crucial Cys residues within Ng molecule that are involved in the conformational changes monitored by the conversion of the 17 k to 10 k species upon non-reducing SDS-PAGE. The reversible conformational changes induced by oxidation could mediate the actions of nitric oxide and other oxidants through changes in the affinity for CaM.

Structure and regulation of the gene encoding the neuron-specific protein kinase C substrate neurogranin (RC3 protein). Neurogranin (Ng/RC3) is a prominent calmodulin-binding protein kinase C (PKC) substrate specifically expressed in the cerebrum of the adult rat brain. Ng/RC3 is expressed in the neurons of the CNS and is especially enriched in neostriatum, neocortex, and hippocampus. Within neurons, this protein is accumulated in the cell bodies and dendrites, where it is localized predominantly in the dendritic shafts and spines. The expression of this gene is relatively low in the fetal and newborn rat brain and an accelerated expression occurs two to three weeks after birth. The cellular and subcellular localizations and the pattern of expression of Ng/RC3 during development, in many respects, resemble those of the PKC γ isozyme; however, the Ng/RC3 gene has a more restricted neuronal expression than PKC γ. A 13-kb genomic DNA encoding Ng/RC3 has been sequenced. The Ng/RC3 gene composes of four exons and three introns with the protein coding region located in the first and second exons. This gene was found to have multiple transcriptional start sites clustered within 20 base pairs (bp); it lacks the TATA, GC, and CCAAT boxes at the proximal upstream region of the start sites. The promoter activity was characterized by transfection of the 293 cells with nested deletion mutants of the 5'-flanking region fused to the luciferase reporter gene. A minimal construct containing +11 to +256 bp was nearly as active as that covering -1508 to +256 bp, whereas a shorter one covering +40 to +256 bp had a greatly reduced activity. Between +11 and +40 bp lies a 12-nucleotide sequence, CCCCGCCCACCC, containing overlapping binding sites for AP2 (CCGCCCACCC) and SP1 (CCCGCC); this region may be important to confer the basal transcriptional activity of the Ng/RC3 gene. The expression of a Ng/RC3-luciferase fusion construct (-1508/+256) in the transfected 293 cells was stimulated by phorbol-12-myristate-13-acetate (PMA), but not by cAMP, arachidonic acid, vitamin D, retinoic acid, nor thyroxine T3 and T4. PMA caused a 2-4-fold stimulation of all the reporter gene constructs ranging from +11/+256 to -1508/+256. The stimulatory effects of PMA could be magnified by co-transfection with both Ca^{2+}-dependent and -independent phorbol ester-binding PKC α, β_I, β_{II}, γ, δ, and ϵ cDNAs but not by the non-phorbol ester-binding PKC ζ cDNA. Ng/RC3 and PKC γ genes have a similar expression pattern in the brain during development. These two genes share at least four conserved sequence segments 1.5-kb upstream of their transcriptional start sites and a gross similarity in possessing several AT-rich segments within -550 to -950bp. A near-homogeneous M_r=20 k DNA-binding protein purified from rat brain was able to bind to these AT-rich regions of both Ng/RC3 and PKC γ genes with footprints containing ATTA, ATAA, and AATA sequences. This DNA-binding protein could be phosphorylated by PKC resulting in an attenuated binding to the DNA.

Binding of myristoylated alanine-rich protein kinase C substrate (MARCKS) to phosphoinositides attenuates the phosphorylation by protein kinase C Stimulation of protein kinase C (PKC) isozymes resulting from ligand-induced turnover of membrane phospholipids is one of the most common signal transduction pathways in eukaryotic cells. Several physiological target substrates of these kinases have been identified and among them the phosphorylation of MARCKS (myristoylated alanine-rich protein kinase C substrate) is synonymous with the activation of PKC *in vivo*. MARCKS is a rod-shaped heat- and acid-stable glycoprotein that reversibly binds to Ca^{2+}/calmodulin, actin filaments, and cellular membrane dependent upon the degree of phosphorylation by PKC. We showed that MARCKS binds to acidic phospholipids with high affinity (K_d less than 0.5 μM) but binds less effectively to neutral phospholipids. Although interaction of MARCKS with acidic phospholipids lacks specificity when determined by binding assay, these phospholipids exert distinctive effects on the phosphorylation of this protein by protein kinase C (PKC). Preincubation of MARCKS with phosphatidylserine (PS) or phosphatidylglycerol (PG) enhanced the phosphorylation; whereas with phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), or phosphatidylinositol-4,5-bisphosphate (PIP_2) inhibited the phosphorylation of this substrate by PKC. Phosphoinositide inhibition of MARCKS phosphorylation was apparently directed at the substrate rather than at the kinase as the

phosphorylation of two other PKC substrates, neuromodulin and neurogranin, exhibited different responses from those of MARCKS. Furthermore, the inhibition of phosphoinositides on MARCKS phosphorylation was seen with PKC isozymes α, β, γ, and δ and with the catalytic fragment of PKC, PKM. The differential effects of PS and PI on MARCKS phosphorylation were also observed with a 25-amino acid synthetic peptide corresponding to the phosphorylation site domain (PSD) of this protein. This peptide, but not the myristoylated N-terminal peptide, competed equally effectively with MARCKS in binding to either PS- or PI-containing vesicles, suggesting that both phospholipids bind to the PSD of MARCKS. Binding of PI to MARCKS retarded PKC phosphorylation of all three phosphorylation sites. The PI-mediated inhibition of MARCKS phosphorylation by PKC was not reversed by PS in a competition experiment, indicating that these two phospholipids bind different residues within PSD. A hypothetical model predicts that the acidic phospholipids could differentially affect MARCKS phosphorylation by binding at separate residues of the PSD, which is situated in the middle of the rod-shaped MARCKS molecule.

Phosphorylation of MARCKS, neuromodulin, and neurogranin by protein kinase C exhibits differential responses to diacylglycerols. Protein kinase C has been implicated in numerous cellular signaling pathways. The pleiotropic regulatory functions of PKC can be explained, in part, by selective tissue, cellular, and sub-cellular distributions of 11 isozymes, α, $\beta 1$, $\beta 2$, γ, δ, ϵ, η, θ, ι, ζ, and μ, which differ in their activation properties and substrate specificities. The 11 members of the PKC family can be divided into 4 groups, group A (conventional), α, $\beta 1$, $\beta 2$, and γ, which are activated by Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DG); group B (novel), δ, ϵ, η, and θ, which are Ca^{2+}-independent, but are activated by PS and DG; group C (atypical), ζ and ι, which are Ca^{2+}- and DG-independent, but are activated by PS; and group D, μ, which is Ca^{2+}-independent but is PS and DG dependent and has an extended N-terminus containing a signal peptide and transmembrane domain. Transient activation of PKC in intact cells is known to be due to DGs generated from phosphoinositide turnover as determined by analysis of precursor phospholipid species and the DGs generated *in situ*. Following the initial rise of DGs derived from hydrolysis of phosphoinositides there is a sustained elevated level of DGs species originating from PC hydrolysis in stimulated cells. A major question is to what extent can DG species generated from PC, as compared to PI, activate the various PKC isoforms with respect to their physiological substrates. We have examined DGs derived from brain PC and PI and synthetic 1,2-dioleoylglycerol ($diC_{18:1}$) and 1,2-dioctanoylglycerol (diC_8) in their ability to activate PKC α, β, and γ using the dominant physiological substrates in the brain, MARCKS, Nm, and Ng. The $A_{0.5}$ of these DGs for PKC were variable dependent on the protein substrates; the values were lowest with MARCKS and highest with Ng. With Ng as a substrate the $A_{0.5}$ of these DGs for PKC γ were PI- and PC-DGs $<$ $diC_{18:1}$ $<$ diC_8. Both PI- and PC-DGs, in spite of their differences in unsaturated fatty acids content, were similarly effective in stimulating PKC. Since the phosphorylation of MARCKS, as compared to those of Nm and Ng, has the lowest $A_{0.5}$ with the various DGs, it seems that among these three PKC substrates MARCKS is most readily phosphorylated by PKCs following DG formation *in vivo*. These results show that the individual PKC substrates have inherent characteristics in distinguishing the various DGs as their preferred activators.

Functional characterization of a novel phosphoprotein HASPP28. The cDNA of a novel phosphoprotein, HASPP28 (for heat and acid stable phosphoprotein of M_r=28k or PP28 for short) has been cloned from a rat brain cDNA library. The cDNA codes for a 180 amino acid polypeptide, which contains identical N-terminal 40 amino acid sequence as the purified rat brain PP28. Antiserum raised against *E. coli*- produced recombinant protein (rPP28) recognizes the purified rat brain PP28, rPP28, and a M_r=28k polypeptide in the extracts of cultured COS, NIH3T3, and N1E115, and N18 neuroblastoma cells. Northern blot analysis showed a single 1.9 kb mRNA species in all the tissues tested (high in brain,

lung. and spleen, moderate in kidney and liver, and low in heart and muscle). Both the purified PP28 and rPP28 are phosphorylatable, as predicted from consensus sequence search, by PKC, PKA, CKI and CKII. Tryptic peptide analyses by 2D-mapping and IEF gel yielded distinct patterns when rPP28 was phosphorylated by these kinases in the presence of [γ-^{32}P]ATP. Anti-PP28 serum immunoprecipitated a M_r=28k phosphoprotein from [^{32}P]PO_4-labeled N1E115 neuroblastoma cells. The tryptic phosphopeptide pattern of the immunoprecipitated protein exhibited similar pattern to that of *in vitro* CKII p phosphorylated rPP28. When cells were treated with TPA or cAMP, which stimulates PKC or PKA, respectively, the immunoprecipitate again yielded a phosphopeptide pattern similar to that of the CKII phosphorylated rPP28. It seems that PP28 is a physiological substrate of CKII or CKII-like kinase even though PP28 is a substrate of PKC and PKA *in vitro*. Phosphopeptides from CKII phosphorylated rPP28 were purified by reverse phase HPLC on C_{18} column and sequenced. More than 95% of the ^{32}P was associated with a peptide, $S^{56}LDS^{59}DES^{62}EDEDDDYQQK^{72}$, mostly with S^{62} and some with S^{59} and S^{56} was devoid of any phosphate. Purified PP28 or rPP28 does not autophosphorylate nor exhibit kinase activity when provided with peptide substrate for CKII, PKC or PKA. The immunoprecipitated PP28 derived from exponentially growing N1E115 cells displayed kinase activity, which was not affected by PS/DG/Ca^{2+}, cAMP, Ca^{2+}/CaM, staurosporin, or PKI, but was inhibited by heparin, a specific inhibitor of CKII. It appears that, in intact cells, PP28 is associated with CKII.

Phosphorylation and protein level of HASPP28 are cell-cycle regulated. Since PP28 is a CKII substrate and CKII is phosphorylated and activated by $p34^{cdc2}$ kinase, a maturation promoting enzyme during the G_2/M transition in the cell cycle, it is predicted that phosphorylation of PP28 is also cell cycle-dependent. Human HeLa cells, just prior to confluence, were synchronized to various stages of the cell cycle: 48h serum deprivation to obtain quiescent cells (G_0); 16h hydroxyurea treatment to obtain actively replicating cells (G_1/S); and 12 h thymidine block and 6 h relief before a 12 h treatment of nocodazole to obtain mitotic cells (G_2/M). These cells were metabolically labeled with [^{32}P]PO_4. Immunoprecipitates of these cells at various stages of cell cycle showed different degrees of phosphorylation; those of mitotic cells had the lowest level of [^{32}P]-labeling and were doublet. Extracts of these cells were also immunoblot analyzed for the levels of PP28; again, the mitotic cells showed the lowest level of PP28 when the same amount of proteins were analyzed, and again they were doublet. The doublet feature of PP28 at the mitotic stage of the cycle may be due to specific phosphorylation at a different site(s) by CKII or by a second kinase. This unique phosphorylation may trigger down-regulation of PP28 at the mitotic stage of the cell cycle. PP28 was found to associate with the particulate fraction of the HeLa cell homogenate prepared in buffer containing low salt; at salt concentrations equal or greater than 150 mM, this protein became cytosolic. The particulate fraction-associated PP28 was not extractable by 0.5% NP40, which normally dissociates the membrane-bound protein. Likewise, the association of exogenously added rPP28 to the particulate fraction of HeLa cells was also sensitive to salt concentration. It seems that PP28 may complex with cytoskeletal unit but not with membrane protein/lipid. As PP28 level is lowest during G_2/M, it would be of interest to determine if PP28 is a subunit of proteasomes (the multi-catalytic protease complex), which functions in down-regulating several key proteins (such as cyclin and cyclin-dependent kinase inhibitor p27) during mitosis.

Roles of cAMP/PKA and TPA/PKC on the neurite outgrowth of N1E115 cells. Morphologically, the neurite outgrowth (NOG) of mouse neuroblastoma N1E115 cells can be induced by 8-Br-cAMP (1 mM, 30 min) or other permeable cAMP analogues and agonists raising cAMP. Treatment of N1E115 cells with TPA, either simultaneously or briefly before the addition of 8-Br-cAMP, resulted in the inhibition of cAMP-mediated NOG. Such inhibition was relieved after prolonged TPA treatment. We have investigated the mechanism of 8-Br-cAMP-mediated NOG/differentiation, and the cross talk between cAMP/PKA and TPA/PKC signaling pathways. Cell extracts prepared from 8-Br-cAMP treated cells, having

differentiated morphology, showed elevated cAMP-independent kinase activity on specific PKA synthetic peptide substrate. On the other hand, extracts prepared from PMA treated cells indicated elevated membrane- and diminished cytosolic-PKC activities. Experiments were designed to identify *de novo* phosphorylated protein(s) under the influence of either activated PKC or PKA in a permeabilized cell system with exogenously added [γ-^{32}P]ATP. Analysis of phosphopeptides on SDS-PAGE showed that cAMP did not, but PMA did promote cellular protein phosphorylation greater than that of non-treated cells. Surprisingly, a protein band having an apparent M_r of 95 k (p95) appeared to be rapidly dephosphorylated under the influence of cAMP, and the dephosphorylation followed a dose-dependent manner. p95 from the non-treated cells was purified by DE52 ion-exchange chromatography and Bio-Gel A-0.5m gel filtration to near homogeneity and was subjected to microsequencing. The N-terminal 15 amino acid sequence was identical to that of eukaryotic elongation factor 2 (eEF-2). It has been established that increased phosphorylation of EF-2, catalyzed by the Ca/CaM dependent kinase III (EF-2 kinase) inhibits translation elongation, while dephosphorylation of EF-2 by protein phosphatase 2A (pp2A) stimulates translation elongation. Since the phosphorylation of p95 in N1E115 cells was enhanced by the presence of okadaic acid (the inhibitor of pp2A), but inhibited by the presence of EGTA and trifluoperazine (inhibitor of Ca/CaM kinase III), the identity of the 95 kDa protein as the eEF-2 is fairly convincing. These results suggest that addition of cAMP promotes dephosphorylation of EF-2 leading to an stimulated protein synthesis machinery. However, neither NOG nor dephosphorylation of p95 is affected by the presence of cycloheximide implying that these early responses do not require any *de novo* protein synthesis. The mechanism is not presently clear how activation of PKA will lead to activation of pp2A (or inactivation of Ca/CaM kinase III) yielding decrease phosphorylation of p95.

Effect of PMA and antisense-inhibition of PKC in the cAMP-mediated NOG of N1E115 neuroblastoma cells. As cAMP-induced NOG in N1E115 cells is blocked by brief treatment with TPA, we are interested in the types of PKC isozymes expressed in these cells and their responses toward short and prolonged treatment of PMA. Immunoblot analysis with isozyme-specific antibodies revealed that PKC α, $\beta 1$, δ, and ζ were present in these cells. After treatment with 1 μM PMA, PKC α and δ were translocated from cytosol to the particulate fraction within minutes, and were downregulated following 4-8 h treatment. In the meantime, cytosolic PKC $\beta 1$ was only partially translocated, and that in the particulate fraction was not downregulated even after 72 h of treatment. PKC ζ-specific antibodies recognize a pair of doublet at 82 kDa. During the PMA treatment, the top band of the doublet was also rapidly translocated and downregulated while the lower band of the doublet remained nonresponsive throughout the 72 h of treatment as would be expected of the authentic PKCζ. As GIBCO's PKCζ antibody was raised with a synthetic peptide that shares some homologies with PKC α, β, and γ, Walsh *et al.*reported that the top band of the doublet probably represents the presence of either individual PKC α, β, and γ, or various combinations of these enzymes. It is not readily understood, however, why the PMA response of the top band of the PKC ζ doublet did not follow those of PKC α and $\beta 1$. Nevertheless, prolonged PMA treatment (24-48 h) completely down-regulated PKC α and δ, correlated with reduced PMA inhibition of the cAMP-mediated NOG, implying that PKC α or δ or both are probably involved in such PMA action. Since PKC $\beta 1$ is not completely downregulated, and PMA inhibition only partially released, a PKC$\beta 1$ antisense strategy was designed. In an stable transfectant of antisense $\beta 1$, where the PKC $\beta 1$ level was greatly reduced, prolonged PMA treatment again downregulated PKC α and δ, and PMA inhibition was almost completely blocked.

Purification and characterization of a novel PKC and p34^{cdc2} kinase substrate p14.4 An *in vitro* PKC substrate from the acid-soluble protein fraction of rat brain was purified to homogeneity by chromatography on DEAE-cellulose, Mono S, and HPLC, and its amino acid sequence was determined. The sequence of this 38-amino acid polypeptide (BP38) is identical to the N-terminal 38-amino acid

region of a 59-amino acid protein (S30) of the rat 40S ribosomal subunit. S30 is derived from a larger precursor, which is composed of a ubiquitin-like protein at the N- and S30 protein at the C-terminus. The ubiquitin-like protein, the 74-amino acid sequence of which predicted from cDNA shares 38% identity with ubiquitin, contains the characteristic C-terminal gly-gly dipeptide of this protein family. It is not clear how the ubiquitin-S30 fusion protein (product of a single mRNA) is processed to give rise to S30 as prepared from 40S ribosome by acid precipitation. It is also unclear how rat brain BP38 is generated. A synthetic peptide corresponding to residues 21-38 of BP38 was used to prepare antiserum (Ab1710), which recognized BP38 and a protein of M_r=14.4k (p14.4) in rat brain extract. Immunoreactive p14.4 is present in all the rat tissues tested (highest in spleen, moderate in brain, kidney, lung, and liver, and low in heart and muscle) and in many cloned cell lines of diverse origins (N1E115, COS, GT1-7, and HeLa cells). It appears exclusively in the cytosolic fractions, which is not expected if it is a ribosomal protein. BP38 contains the sequence motif for PKC phosphorylation, SXXR, and that for $p34^{cdc2}$ kinase, TPK, and the protein is an excellent substrate for both kinases. As BP38 may be derived from p14.4 during purification under acidic conditions, a gentle procedure was developed to isolated the native protein. A highly purified p14.4 was prepared from the high-speed supernatant of rat brain extract by chromatography on DEAE-cellulose, phospho-cellulose, and hydroxylapatite. A pair of primers corresponding to S30 cDNA were used to amplify S30 cDNA by RT-PCR using rat kidney RNA. A predicted 474 bp product was cloned directly into pCRII and sequence verified. Two forward primers were designed each carried a Nde site at the 5'-end; an F1 primer for the cDNA to generate the ubiquitin-S30 fusion protein and an F2 primer for cDNA to make S30. In both cases, an SP6 oligo was used as the reverse primer. Both PCR products of the predicted size were doubly digested with Nde and EcoR1 and cloned into the pRSETc vector linearized by the same restriction enzymes. pRSETc-F1 and pRSETc-F2 were transformed into E. coli BL21-DE3 for recombinant protein production. Neither recombinant F1 (ubiquitin-S30 fusion protein) nor F2 (S30 protein) co-migrate with p14.4 on SDS-PAGE following immunoblot analysis. All three proteins, F1, F2, and p14.4, were degraded to smaller fragments upon exposure to 2% perchloric acid at 0°, indicating that this protein is highly labile.

Significance to Biochemical Research and the Program of the Institute:

It is the aim of this project to investigate the mechanisms by which hormones, neurotransmitters, and growth factors regulate cellular functions. It is generally believed that the ligand-receptor interaction triggers the release of second messenger(s) which affect(s) the activities of key enzymes through covalent modification of proteins. The signaling systems using cAMP, Ca^{2+}/calmodulin, and Ca^{2+}/diacylglycerol frequently cause changes in the state of phosphorylation and that using nitric oxide causes changes in the state of oxidation of proteins. All these signaling pathways regulate every aspect of living processes including growth, differentiation, metabolism, sensory transduction, immune response, and learning and memory. Understanding the mechanisms of action of these second messengers in controlling the various cellular responses will lead to the development of effective therapy for the related diseases. Our original work on the Ca^{2+}/diacylglycerol/PKC pathway that led to the discovery of the molecular diversity of this enzyme family has greatly enhanced our understanding of the role of this enzyme in a variety cellular responses.The second messenger role of diacylglycerol in the activation of protein kinase C can be mimicked by tumor-promoting phorbol esters. Protein kinase C has been identified as a receptor for these tumor promoters and is thought to mediate the pleiotropic actions of these compounds. We have investigated the role of PKC isozymes in the regulation of cell differentiation using N1E115 neuroblastoma cell neurite-outgrowth model. Phosphorylation of target substrates by PKC is the first step leading to cellular responses. A group of calmodulin-binding PKC substrates, including neurogranin, neuromodulin, and MARCKS, have been shown to be physiological targets of the kinase. Phosphorylation of these proteins by PKC favors the release of calmodulin which becomes available for many calmodulin-

dependent enzymes. This signal amplification step is essential to transduce the extracellular signals for the pleiotropic cellular responses. In order to further define the mechanism by which PKCs transduce the external signal, we investigated the presence of other potential target substrates of PKC in the brain. As a first step for systematic analysis of these proteins, we purified several acid-stable proteins as potential substrates of PKC. Amino acid sequence analysis of these proteins revealed several novel PKC substrates. This group of perchloric acid-soluble proteins contained prominent PKC substrates, neurogranin, neuromodulin, and MARCKS, as well as many nuclear proteins, such as histones and high-mobility group chromosomal proteins. We have cloned the cDNA of a novel M_r=28k phosphoprotein having a broad tissue distribution. This protein has been shown to be an *in vivo* substrate of casein kinase II and both the state of phosphorylation and protein level are cell cycle-regulated. The discovery and cloning of this novel PKC/casein kinase II substrate will lead to a new research area related to the PKC/casein kinase II-mediated signaling pathway. In addition, we have cloned another M_r=14.4k protein, which is a potential substrate of both PKC and mitogen-activated $p34^{cdc2}$ kinase. The sites of phosphorylation of this protein by PKC and $p34^{cdc2}$ kinase have been determined and their phosphorylations during cell cycle will be investigated.

Among the PKC substrates studied, we have identified neurogranin as a potential target of nitric oxide and other oxidants. One of the most intriguing findings of our study is the discovery of the oxidation/reduction-mediated regulation of neurogranin and calmodulin interaction. Oxidation of neurogranin by nitric oxide or hydrogen peroxide forms intramolecular disulfide bond resulting in gross conformational changes and thus reducing the affinity for calmodulin. The oxidized neurogranin can be reduced by sulfhydryl reagent to regenerate the high affinity calmodulin-binding protein. As calmodulin is an essential component for the regulation of numerous enzymes, including several calmodulin-dependent protein kinases, adenylyl cyclase, nitric oxide synthase, Ca^{2+} pump, protein phosphatase, cAMP phosphodiesterase, and IP_3 kinase, this newly discovered signaling mechanism involving reversible oxidation/reduction to control the affinity for calmodulin could be involved in many physiological responses. In the CNS, the excitatory amino acid-mediated stimulation of NMDA receptor that leads to the generation of nitric oxide could increase the free calmodulin level in the target cells. This mechanism functions in concert with the excitatory amino acid-mediated increase in $[Ca^{2+}]_i$, turnover of membrane phospholipids generating lipid second messengers (diacylglycerol), and activation of PKC that enhances the phosphorylation of neurogranin. Since an increase in $[Ca^{2+}]_i$, generation of nitric oxide, and activation of PKC have been linked to numerous neuronal functions including enhancement of neuronal plasticity, it is likely that the release of calmodulin from the calmodulin-binding PKC substrates may participate in the learning and memory as demonstrated in the long-term potentiation model..

Neurogranin is specifically expressed in a limited number of neurons of the adult brain. This protein is accumulated in the cell bodies and dendritic shafts and spines during development. Phosphorylation of this protein by PKC has been linked to the control of ion channel and long-term potentiation and is thus likely to be involved in learning and memory in higher organisms. Study of the expression of this gene could unravel the transcriptional mechanism that regulates the cell type-specific and developmental stage-regulated expression. We have identified a genomic clone harboring the entire coding region of this gene and have characterized its promoter region. The promoter activity of this gene is stimulated by phorbol ester and by co-transfection with several PKC cDNAs, suggesting the involvement of PKC in transcriptional activation. This finding indicates that activation of PKC could trigger the expression of its substrate. In order to dissect the transcriptional machinery of this gene we have isolated an AT-rich DNA-binding protein that interacts with sequence elements containing ATTA, ATAA, or AATA. This protein also interacts with the PKC γ gene at a location similar to that of neurogranin. We have also identified this protein as a substrate of PKC and shown that its binding affinity is greatly reduced upon

phosphorylation. Both PKC γ and neurogranin genes are expressed selectively in neurons in a parallel fashion during development. These two genes may share certain common regulatory mechanism. Elucidation of the genomic structures of PKC γ and neurogranin genes will facilitate the investigation into the tissue-specific and development-regulated expression of these proteins. Both proteins are expressed at high levels in the cerebral cortex, hippocampus, and amygdala of adult rat brain. Studies on the expression and turnover of these proteins in the nervous system may provide clues to the normal and pathological development related to mnemonic function.

Proposed Course:

This project represents an integrated and comprehensive approach to understand the control of cellular functions by reversible covalent modification of proteins. Both the phosphorylation and oxidation are the major focus of this study. Because protein kinase C and its down-stream target substrates have been implicated in the signal transduction to regulate many physiological processes, we will continue to focus our efforts on studying the physicochemical properties of the various protein kinase C isozymes and their substrates; characterization of the physiological functions of the various PKC substrates; identification of novel PKC substrates by protein purification and molecular cloning; and characterization of the regulatory mechanism for the expression of PKC and its substrates.

The discovery of Ng as a redox-sensitive calmodulin-binding protein raises many interesting questions concerning whether this regulatory process occurs *in vivo*. An antibody against rat brain Ng has been prepared and will be used to detect the nitric oxide-induced modification of this protein. Because of the ease of detection of Ng oxidation by SDS-PAGE and immunoblot, this system will became very useful for characterizing the nitric oxide signaling mechanism in CNS. Since both the phosphorylation and oxidation of Ng reduce its binding affinity to calmodulin, it is likely that the excitatory amino acid-induced activation of PKC and production of nitric oxide may affect the cellular responses through modifications of Ng. Stimulation of neurons by excitatory amino acids that induces long-term potentiation or neurotoxicity can be monitored by following the changes in Ng molecular weight. Effect of nitric oxide in the modification of Ng in intact cells will also be tested in the wild type and mutant Ng cDNA-transfected cell lines. Fluorescence probe-labeled protein will be injected into neurons grown in culture to determine the fate of this protein upon stimulation of PKC and release of nitric oxide. Currently, Ng cDNA has been cloned from mouse and rat, both have identical deduced amino acid sequence. We will attempt to clone the cDNAs from human and from lower organisms to determine the phylogenetic conservation of the important Cys residues during evolution. As Ng is a highly potent reductant capable of serving an acceptor of nitric oxide, a homologous molecule is likely present in other tissues such as vascular smooth muscle, which response to nitric oxide for relaxation, and in macrophages, which generate large dose of nitric oxide for tumoricidal and bactericidal actions. The biological function of Ng in the brain will also be tested with mutant mice, whose Ng gene is disrupted.

Ng from rat brain and recombinant wild type and mutant proteins have been prepared in milligram quantity. The structure feature of this protein that endowed with potent reducing power will be explored by studying its three dimensional structure by NMR and X-ray crystallography. The reduced, oxidized, phosphorylated, and phosphorylated/oxidized Ng will be used. Further mutagenesis study to define the structural features of Ng as a potent reductant will be investigated. This protein possesses certain structural features of those neurotoxins capable of modulating ion channels. The functional role of this protein will be tested for its ability to modulate ion channels by patch-clamp analysis with whole-cell and single channel patch. Wild type and mutant Ng in reduced, oxidized, PKC-phosphorylated, and phosphorylated/oxidized forms will be tested for their effects on the various cationic and anionic channels.

Both phosphorylation/dephosphorylation and oxidation/reduction of Ng influence the binding of Ng to calmodulin that serves as signal amplification steps of diacylglycerol and nitric oxide second messenger systems. It is of interest to determine the reversal of PKC phosphorylation by protein phosphatase and nitric oxide oxidation by either chemical or enzymatic reduction. Previously, we have shown dephosphorylation of Ng by protein phosphatase 1, 2A, and 2B; it has yet to determine if the phosphorylated/oxidized Ng can be effectively dephosphorylated by these phosphatases. Reduction of the oxidized Ng occurs *in vitro* by DTT; we have yet to determine the reduction potential of Ng oxidation and the effectiveness of biological reductant, such as glutathione, to reduce the oxidized Ng. It is also possible that an enzyme similar to glutathione reductase that utilizes NADPH for reduction of Ng. Oxidation of Ng by nitric oxide generates nitroso-Ng intermediate which may serve as a donor of nitric oxide for other acceptors, such as glutathione and other proteins.

The expression of PKC γ and Ng in the brain is believed to be developmentally controlled. The factor that governs the development- and tissue-specific expression of these two genes must be located in the promoter region. We have already sequenced the 5'-flanking region of the PKC γ and Ng genes and detected several potential specific DNA-binding sites in the promoter region. Since the control of neurogranin gene expression is similar to that of PKC γ, we anticipate that both genes possess similar regulatory elements. Comparison of the structure features between these two genes may shed some light on their common regulatory mechanism. The DNA-binding proteins for these promoters will be isolated to define their modes of action, in particular, with respect to the stimulatory effect of PKC on gene expression. As the genomic structure of Ng has been characterized, we will employ the transgenic mice model to define the tissue and cell type-specific expression of this gene.

Identification of target substrates of a protein kinase is essential to understand the signaling mechanism of a second messenger system. A novel M_r=28k rat brain protein kinase C substrate has been purified to near homogeneity and its cDNA cloned. This protein is an *in vivo* CKII substrate and the phosphorylation and protein level were regulated in a cell cycle-dependent manner. However, the functional role of this protein is unknown. Attempt will be made to determine i) if the association of PP28 with CKII is cell cycle-dependent and the phosphorylation affects PP28/CKII association; ii) the cause of specific phosphorylation that results in the doublet formation in mitotic cells; iii) if this specific phosphorylation serves as a signal for downregulation of PP28 at mitotic stage; iv) whether the degradation of PP28 is carried out through the ubiquitin-proteasome pathway; and v) a putative receptor protein of PP28 in the detergent-insensitive but salt-sensitive particulate fraction of the cells. We are also constructing i) expression plasmids for producing recombinant proteins in bacteria of site-directly mutated PP28, namely, S62A, S62C, and S59AS62A, so to demonstrate that phosphorylation of S59,which is not a CKII site, is a consequence to the phosphorylation of S62; ii) mammalian expression plasmids with PP28 (both wild type and mutated ones) fused N-terminally to a green fluorescent protein (GFP) so to follow the whereabouts of the protein during the cell cycle progression and upon cell stimulation. The M_r=28k protein contains a highly acidic region within which the two CKII phosphorylation sites were located. Phosphorylation of these two Ser residues leads to the presence of a stretch of 11 consecutive acidic amino acids. This region may be involved in the phosphorylation-dependent binding of metal ions or basic proteins.

In the N1E115 cell differentiation model, prolonged PMA treatment and PKC $\beta1$ antisense inhibition down-regulate PKC α, $\beta1$, and δ to a minimum level, yet PMA inhibition of cAMP-mediated NOG is not completely relieved. It is possible that the remaining PKC activity or a non-PKC but PMA responsive component is responsible for the residual inhibition. There is also the PMA-non-responsive PKC ζ, which is constitutively active and which may block the cAMP effect. We plan to stably cotransfect both

antisense β1 and antisense ζ, and to select clones with reduced expression of both PKC β1 and ζ. Again with prolonged PMA treatment, we expect to find all the known PKC isozymes in N1E115 cells at their minimum levels. By this strategy, we hope to study the antagonistic mechanism that PKC exerts on the cAMP induction of NOG, which will eventually aid us in probing the mechanism of NOG induction by cAMP. Identification of the dephosphorylating p95 as eEF-2 demonstrated that activation of protein synthesis is essential in cAMP-mediated neuronal differentiation, but it may or may not play a role in the immediate response of NOG. It is worthwhile to investigate the mechanism that leads from the activation of PKA to the enhancement of PP2A, which is opposite to the usual finding. It is possible that the kinase and phosphatase cascades operate slightly differently in these neuroblastoma cells than in other cell systems. Previously, we demonstrated that the norepinephrine release from N1E115 cells was strongly enhanced by depolarization (K$^+$), Ca^{2+} (Bay K 8644), carbachol (M1 muscarinic receptor agonist specifically), etc. The release was also stimulated by the presence of PMA, the PKC activator, and inhibited by staurosporin, the PKC inhibitor. Attempts to further enhance the PMA effect by either transiently or stably overexpressing PKC β1 failed. We plan to develop other permanent transfectants expressing other PKC isozymes to study the involvement of PKCs in the enhancement of neurotransmitter release.

p14.4 is a potential substrate of both PKC and p34^{cdc2} kinase and the phosphorylation of this protein may play a role in integrating the signals from both pathways. Native p14.4 from rat brain has been extensively purified, but not yet to homogeneity. This protein needs to be further purified and its sequence determined so to find out whether it could be the native protein of BP38 (and S30). Recombinant F1 and F2 will be produced and purified so that their relationship with p14.4 can be studied. Phosphorylation of p14.4, F1 and F2 by both PKC and p34^{cdc2} and their regulation will be investigated. As p34^{cdc2} is a mitotically active kinase, the phosphorylation status of p14.4 at various stages of cell cycle will be surveyed so to determine whether its phosphorylation is cell cycle-dependent and if it has a role in cell cycle regulation.

Publications:

Mahoney C W, Huang K-P. Selective phosphorylation of cationic polypeptide aggregated with phosphatidylserine/diacylglycerol/Ca^{2+}/detergent mixed micelles by the C^{2+}-independent but not Ca^{2+}-dependent protein kinase C isozymes. Biochemistry 1995;34:3446-3454.

Mahoney C W, Seki K, Huang K-P. Phosphorylation of MARCKS, neuromodulin, and neurogranin by protein kinase C exhibits differential responses to diacylglycerols. Cellular Signalling (in press).

Sato T, Xiao D-M, Li H, Huang FL, Huang K-P. Structure and regulation of the gene encoding the neuron-specific protein kinase C substrate neurogranin (RC3 protein). J Biol Chem 1995;270:10314-10322.

Seki K, Chen H-C, Huang, K.-P. Dephosphorylation of protein kinase C substrates, neurogranin, neuromodulin, and MARCKS, by calcineurin and protein phosphatase 1 and 2A. Arch Biochem Biophys 1995;316:673-679.

Sheu F-S, Huang FL, Huang K-P. Differential responses of protein kinase C substrates (MARCKS, neuromodulin, and neurogranin) phosphorylation to calmodulin and S100. Arch Biochem Biophys 1995;316:335-342.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00193-10 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Angiotensin II Receptors and Activation Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Structure-function studies on the AT_1 receptor that mediates most of the known actions of angiotensin II (Ang II) have revealed further aspects of its ligand binding site and the domains involved in receptor activation, signaling, and internalization. The amino acid residues required for binding of the non-peptide antagonist, losartan, are confined to the transmembrane domains of the receptor. In contrast, those involved in binding of peptide antagonists and Ang II itself are known to be located largely within extracellular regions of the receptor. Although the losartan binding site has been proposed to be distinct from that of Ang II, at least three transmembrane residues (Arg^{169}, Lys^{199}, and Phe^{301}) participate in binding of both Ang II and losartan. Thus, peptide ligands such as Ang II interact with the intramembrane binding pocket as well as with extracellular residues that subserve high-affinity binding. Non-peptide antagonists block receptor activation by occupying an intramembrane site that overlaps with the the space occupied by the agonist, or by inducing conformational changes that prevent agonist binding. Agonist-induced internalization of the Ang II receptor was found to depend on a cytoplasmic Ser-Thr-Leu motif that is conserved in the carboxyterminal tail of AT_1 receptors but is absent from the non-internalizing AT_2 receptor. The conserved Arg-Pro-x-x-Tyr motif located in the seventh transmembrane domain of most G protein-coupled receptors, which is an internalization signal in many growth factor receptors, does not control endocytosis of the AT_1 receptor. However, the Phe^{301} residue contributes significantly to agonist binding and Asn^{298} is required for normal receptor activation and signal transduction. A tyrosine residue in the fifth transmembrane domain that is highly conserved among G protein-coupled receptors was found to be essential for receptor activation and may be of general importance in signal transduction by seven transmembrane domain receptors. Studies on receptor-mediated signaling responses showed that the potentiation of agonist-induced cAMP production in bovine glomerulosa cells is prevented by inhibitors of the calcium/calmodulin-regulated protein phosphatase, calcineurin. In cultured bovine glomerulosa cells, Ang II stimulated growth responses including activation of MAP kinase and early gene expression, as well as increased thymidine uptake and cell proliferation. The recently discovered ability of the anti-inflammatory agent wortmannin to prevent sustained inositol phosphate and calcium signaling responses to Ang II and other calcium-mobilizing hormones was found to result from inhibition of a novel phosphatidylinositol 4-kinase that is essential for the maintenance of the agonist-sensitive phosphoinositide pools in several cell types.

Project Description

Objectives:

The aims of this project are to characterize the cell-surface receptors for angiotensin II (Ang II) and to define the signal transduction pathways involved in the stimulation of target-cell responses such as activation of aldosterone secretion during Ang II action. Studies include the analysis of radioligand binding properties of membrane-bound and solubilized Ang II receptors; the isolation and characterization of photolabeled Ang II receptors; cloning of cDNAs for mammalian and amphibian Ang II receptors; characterization of endogenous and expressed mammalian Ang II receptors in *Xenopus* oocytes and transfected cells; mutational analysis of Ang II receptors to identify amino acids involved in ligand binding, receptor activation and G protein coupling, and endocytosis of agonist-receptor complexes; investigation of the mechanisms and role of calcium entry and mobilization during Ang II action; analysis of the changes in phospholipid turnover and inositol polyphosphate metabolism that mediate the cellular actions of Ang II in adrenal glomerulosa and other angiotensin target cells; and investigation of the signaling pathways through which Ang II controls growth and proliferation of adrenal glomerulosa cells.

Methods Employed:

Radioligand binding studies with Ang II agonist, antagonist, and photoreactive derivatives; solubilization and fractionation of free and radiolabeled receptor sites; fluorescence analysis of cytoplasmic calcium levels in cell suspensions and single cells; measurement of calcium release from intracellular stores and subcellular fractions; HPLC and TLC analysis of phosphoinositides and inositol phosphates; radioligand studies of intracellular $InsP_3$ receptors; radioimmunoassays of aldosterone, pregnenolone and cyclic nucleotides; expression of adrenal and other mRNAs in *Xenopus* oocytes; Northern, Southern and Western blots; construction of expression libraries; polymerase chain reaction; molecular cloning and sequencing of Ang II receptor subtypes. Transient and stable transfection of mammalian cells with receptor cDNAs.

Major Findings:

Structure-function studies on AT_1 receptors: The availability of cloned Ang II receptors has facilitated the analysis of structural features that are responsible for, or related to, many of the specific functional properties of the AT_1 receptor. We have performed mutational analysis of AT1 receptors to identify amino and sequences and residues that are involved in the processes of ligand binding, agonist activation G protein coupling, and internalization of agonist-receptor complexes.

1. Structural basis of ligand binding to AT_1 receptors. Angiotensin II is a major regulator of blood pressure, aldosterone secretion, and fluid homeostasis, and is also an important etiological factor in arterial hypertension and cardiovascular disease. Several novel nonpeptide Ang II antagonists have been introduced for the treatment of hypertension and are exemplified by biphenylimidazole compounds such as losartan, which binds to AT_1 receptors with high affinity. Losartan and other such AT_1-selective nonpeptide antagonists block all of the known physiological actions of Ang II, and are expected to be widely utilized as antihypertensive agents. The AT_1 receptors of several mammalian and other species consist of single polypeptide chains containing the seven transmembrane domains typical of G protein-coupled receptors (GPCRs). The amphibian Ang II receptor was previously found to be functionally similar to the mammalian AT_1 receptor in that it activates the phospholipase C/calcium signal transduction

pathway, but differs in its pharmacology and does not recognize losartan and other nonpeptide antagonists that bind to the mammalian AT_1 receptor. This property was utilized to identify amino acid residues involved in the binding of losartan to mammalian receptors by analyzing the ligand binding properties of mutant rat AT_1 receptors in which non-conserved amino acids were replaced by the corresponding amphibian residues. Most of these mutant receptors retained normal binding affinity for Ang II and its peptide antagonist [Sar^1,Ile^8]Ang II, indicating that the overall conformation of the receptor was unaltered. However, several residues located in the transmembrane domains of the receptor were found to be required for binding of the nonpeptide antagonist. These findings demonstrated that the nonpeptide AT_1 antagonist binds to a site defined by amino acids located within the membrane-spanning regions of the receptor, and which is largely distinct from the region of the receptor that is involved in binding of Ang II and other peptide ligands.

Further studies have identified an additional six amino acid residues in the transmembrane domains of the rat rAT_1 receptor that are important in losartan binding. When the thirteen residues required for losartan binding in the mammalian receptor were exchanged for the corresponding amino acids in the *Xenopus* AT receptor, the mutant amphibian receptor bound losartan with the same high affinity (2 nM) as the rat rAT_1 receptor. This represents a novel type of gain-of-function mutant in which the residues crucial to formation of a ligand binding site in a peptide hormone receptor have been transferred to a previously unresponsive receptor by site-directed mutagenesis. Alanine scanning and comparison of mammalian and amphibian combinatorial mutants indicated that the third transmembrane domain in the rAT_1 receptor plays a key role in losartan binding. Identification of the residues involved in nonpeptide binding will facilitate studies aimed at elucidating the chemical basis for ligand recognition in the Ang II receptor and peptide hormone receptors in general.

2. *Receptor activation and endocytosis.* Many plasma-membrane receptors become clustered in clathrin-coated pits and are subsequently internalized in clathrin-coated residues to undergo degradation or recycling. While nutrient receptors (e.g., for low density lipoprotein and transferrin) commonly exhibit constitutive internalization, hormones and growth factor receptors that evoke intracellular signals usually undergo ligand-induced internalization. Both growth factor receptors with intrinsic tyrosine kinase activity and G protein-coupled receptors are internalized by a similar clathrin-coated vesicular endocytic process. However, the extent to which the intracellular signaling mechanisms activated by these receptors have a role in the internalization process has not been determined.

To address this question, the relationship between angiotensin II-induced activation of G proteins and receptor internalization was analyzed by transiently expressing mutant and wild type cDNAs for the rat AT_{1a} receptor in COS-7 cells. Pertussis toxin-sensitive G proteins did not appear to play a role in endocytosis since the receptor showed normal internalization kinetics in pertussis toxin-treated cells. Three deletion mutants of the third cytoplasmic loop revealed that the N-terminal part of this region is important for both receptor endocytosis and intracellular signaling. Three point mutants of Asp^{74}, which has been implicated in signal transduction by the AT_1 receptor, caused impaired G protein coupling and inositol phosphate responses. However, each of these mutants (D74N, D74H, and D74Y) showed markedly different internalization kinetics. The D74Y mutant showed the greatest impairment of internalization but retained the highest degree of inositol phosphate stimulation. In contrast, the D74N mutant, which showed the most impaired G protein coupling and inositol phosphate responses, had similar internalization kinetics to the wild type receptor. The combined mutant receptor containing the D74N substitution and deletion of residues 221-226 from the third cytoplasmic loop showed no G protein coupling or inositol phosphate response but was internalized about 60% as rapidly as the wild type receptor. These data demonstrate that endocytosis of the AT_1 receptor is independent of agonist-activated

signal transduction and indicate that receptor internalization and activation of phospholipase C have different structural requirements.

The demonstration that coupling to heterotrimeric G proteins and intracellular signaling responses is not required for internalization of the AT_1 receptor indicated that the conformational changes that result from agonist-induced receptor activation exert largely independent effects on signal transduction and receptor endocytosis. However, since the structural requirements for signaling and internalization overlap, mutations that cause changes in G protein coupling are frequently accompanied by impairment of endocytosis. Thus the N-terminal region of the third cytoplasmic loop is important for both receptor signaling and internalization. However, the ability of internalization-deficient receptors with mutations of non-conserved regions in the cytoplasmic tail or a third intracellular loop to activate their respective G proteins and signaling responses indicates that G protein activation alone is not sufficient to induce receptor endocytosis. The nature of the sequences in these regions that are important for receptor internalization is not well defined, but they are commonly enriched in serine and threonine residues. In the rat AT_1 receptor, 12 serine/threonine residues are present in the 23-amino acid segment located between residues 326 and 348 of the carboxyterminal cytoplasmic tail. Studies on the rat AT_1 receptor showed that its carboxy-terminal 22 amino acids are not necessary for agonist-induced endocytosis. However, receptor internalization were markedly impaired by removal of one additional residue (Leu^{337}), and was reduced by 95% after removal of the adjacent residues, Ser^{335} and Thr^{336}.

Single alanine replacements of amino acids in the region showed that individual substitutions of Thr^{332}, Ser^{335}, Thr^{336}, Leu^{337}, and Ser^{338} caused moderate but significant impairment of the internalization rate. Replacement of both Ser^{335} and Thr^{336} with alanine residues further impaired the internalization rate, and triple alanine replacement of the Ser-Thr-Leu motif reduced internalization to almost the same extent as the corresponding tail deletion mutant. The Ser-Thr-Leu motif is highly conserved in mammalian AT_1 receptors but is not present in the non-internalizing type 2 angiotensin II receptor. These data demonstrate that a serine/threonine-rich region including Leu^{337} in the cytoplasmic tail of the AT_1 receptor is a major requirement for endocytosis of the hormone-receptor complex, and support the support the concept that similar motifs in other G protein-coupled receptors are determinants of their agonist-induced internalization.

3. *Receptor activation and signal transduction.* The AT_1 receptor has been reported to interact with several G proteins, including G_q, G_i, and G_o, but its major physiological functions are expressed through G_q-mediated activation of phospholipase C followed by phosphoinositide hydrolysis and Ca^{2+} signaling. Although all GPCRs possess the basic seven-transmembrane structure, only a few amino acids highly conserved among the super-family of G protein-coupled receptors. One such conserved motif is the characteristic $NPX_{2-3}Y$ sequence that is located in the seventh transmembrane domain of most receptors, and in the rat smooth muscle AT_1 receptor is Asn^{298}-Pro^{229}-Leu^{300}-Phe^{301}-Tyr^{302}. Several G protein-coupled receptor models have been based on the structure of bacteriorhodopsin, which shares no sequence homology with mammalian G protein-coupled receptors but has an identical folding pattern and functional resemblance to mammalian opsins. The recently reported low resolution structural image of bovine rhodopsin has made it possible to further improve these models. A recent model of aminergic G protein-coupled receptors suggests that the $NPX_{2-3}Y$ sequence is ideally placed to receive a signal from agonist-induced conformational changes in the ligand binding region. This sequence is in close proximity to the functionally important asparagine-aspartic acid pair located in transmembrane segments 1 and 2 and may participate in important hydrogen bonding interactions. Most models also predict that the highly conserved proline residues disrupt the α-helical structure of the transmembrane domains and serve as hinges that participate in the agonist-induced conformation change of G protein-coupled receptors. One

such proline residue is located within the seventh transmembrane domain in the conserved NPX$_{2\text{-}3}$Y sequence, and this residue (Pro$_{540}$) of the m$_3$ muscarinic receptor has been found to be important for signal transduction.

Another interesting feature of the NPX$_{2\text{-}3}$Y sequence is its similarity to the NPXY internalization sequence that was first described in the cytoplasmic segment of the low density lipoprotein receptor. In recent studies, the tyrosine residue in this sequence was found to be essential for sequestration of the β-adrenergic receptor but not for the internalization of the gastrin-releasing peptide receptor. The gastrin-releasing peptide receptor and the AT$_1$ receptors, unlike the β-adrenergic receptor, contain an additional aromatic amino acid (Phe^{321} and Phe^{301}, respectively) in their NPX$_{2\text{-}3}$Y sequences. The presence of such Phe residues might be important since phenylalanine can substitute for the tyrosine residue in the NPXY internalization sequence of nutrient receptors.

The role of this sequence (NPLFY) in angiotensin II receptor function was studied in mutant and wild-type rat type 1 angiotensin II receptors transiently expressed in COS-7 cells. The ability of the receptor to interact with G proteins and to stimulate inositol phosphate responses was markedly impaired by alanine replacement of Asn^{298} and was reduced by replacement of Pro^{299} or Tyr^{302}. The F301A mutant receptor exhibited normal G protein coupling and inositol phosphate responses, and the binding of the peptide antagonist, [Sar^1,Ile^8]angiotensin II, was only slightly affected. However, its affinity for angiotensin II and the nonpeptide antagonist losartan was reduced by an order of a magnitude, suggesting that angiotensin II and losartan share an intramembrane binding site, possibly through their aromatic moieties. None of the agonist-occupied mutant receptors, including Y302A and triple alanine replacements of Phe^{301}, Tyr^{302}, and Phe^{304}, showed substantial changes in their internalization kinetics. These findings demonstrate that the NPLFY sequence of the type I angiotensin II receptor is not an important determinant of agonist-induced internalization. However, the Phe^{301} residue contributes significantly to agonist binding, and Asn^{298} is required for normal receptor activation and signal transduction.

Another highly conserved amino acid in the superfamily of G protein-coupled receptors is a tyrosine residue located in the fifth transmembrane helix, adjacent to the amino-terminal end of the third cytoplasmic loop. The location of this amino acid, which is Tyr^{215} in the rat AT$_1$ receptor, suggests that it might play an important role in receptor activation. The amino-terminal region of the third cytoplasmic loop adjacent to this residue has been shown to be important in the signal generation and internalization of several G protein-coupled receptors. Furthermore, modeling of G protein-coupled receptors based on the low resolution crystal structure of bovine rhodopsin molecule has suggested that this residue is in molecular proximity to regions that are important in receptor activation, including the conserved acidic-arginine-aromatic (DRY) triplet of the second intracellular loop. We have shown previously that AT$_1$ receptor internalization and signal transduction have different structural requirements. However, a 6-amino acid deletion of the amino-terminal end of the third cytoplasmic loop, which included Tyr^{215}, prevented both AT$_{1a}$ receptor internalization and signaling responses. To evaluate the role of the highly conserved Try^{215} residue in the activation process of G protein-coupled receptors, we created a mutant receptor by substituting Tyr^{215} with phenylalanine (Y215F). This mutant receptor cDNA was transfected into COS-7 cells and its binding, internalization, and signaling properties were compared with those of the wild type AT$_1$ receptor.

The mutant receptor was highly expressed in transfected COS-7 cells, and its binding affinity for the peptide antagonist [Sar^1,Ile^8]Ang II was similar to that of the wild type receptor. Although the structural integrity of the peptide ligand binding domain was preserved in the Y215F mutant receptor, its affinity

for the native agonist, Ang II, was significantly reduced. Also, whereas guanosine 5'-3-O-(thio)triphosphate markedly reduced Ang II binding to the wild type receptor, it had little effect on agonist binding to the mutant receptor. Agonist-induced internalization of the mutant receptor was also impaired, and its ability to mediate inositol phosphate responses to Ang II stimulation was abolished. The concomitant decreases in receptor internalization and G protein-mediated signaling of the Y215F mutant receptor indicate that Tyr^{215} has a critical role in AT_1 receptor activation. In view of its conservation among members of the seven transmembrane domain receptor superfamily, this residue is likely to be of general importance in signal transduction from G protein-coupled receptors.

A novel phosphatidylinositol 4-kinase essential for agonist-induced $InsP_3$/calcium signaling: In many agonist-stimulated cells, the initial increases in both $Ins(1,4,5)P_3$ production and $[Ca^2]_i$ are temporally correlated and are largely independent of extracellular Ca^{2+}. These responses are consistent with the activation of phospholipase C to produce $Ins(1,4,5)P_3$ which in turn binds to the $Ins(1,4,5)P_3$ receptor channel and promotes Ca^2 release from the endoplasmic reticulum. The sustained increase of $[Ca^{2+}]_i$ is caused by Ca^{2+} influx from the extracellular medium, and the secondary increase of $Ins(1,4,5)P_3$ formation is largely dependent on the same Ca^{2+} influx. This close relationship between the sustained rises in $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ complicates the analysis of their respective roles during this phase of the agonist-induced signaling response. However, it is generally agreed that $Ins(1,4,5)P_3$, acting directly on the plasma membrane and/or indirectly by emptying intracellular Ca^{2+} stores, activates a Ca^{2+} entry pathway that maintains the second phase of the cytoplasmic Ca^{2+} response. In many cells, the secondary increase in $[Ca^{2+}]_i$ appears to exert positive feedback on phospholipase C to promote its activity and maintain the formation of $Ins(1,4,5)P_3$.

We recently observed that the fungal metabolite, wortmannin (WT), a potent inhibitor of myosin light chain kinase, caused dose-dependent inhibition of the sustained but not the initial increases in $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ in angiotensin II (Ang II)-stimulated adrenal glomerulosa cells. However, WT did not affect the $[Ca^{2+}]_i$ increases evoked by K^+-induced depolarization or by depletion of intracellular Ca^{2+}-stores with ionomycin or thapsigargin, suggesting that its inhibitory effects on $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ responses are closely related. The inhibition of Ca^{2+} influx by WT was found to be a consequence of reduced $Ins(1,4,5)P_3$ formation and could reflect the resultant refilling of the agonist-sensitive Ca^{2+} pool, since Ang II-depleted Ca^{2+} pools were found to refill by a thapsigargin-sensitive mechanism in WT-treated cells. These findings demonstrated that WT is a potent inhibitor of the $Ins(1,4,5)P_3$-Ca^{2+} signaling pathway and acts primarily on a mechanism that is required for $Ins(1,4,5)P_3$ formation during sustained agonist action.

Further exploration of the site of action of WT was performed in adrenal glomerulosa cells and other cell types, and focused on the pathway responsible for biosynthesis and maintenance of phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$] the immediate precursor of intracellular signals generated by calcium-mobilizing hormones and growth factors. The synthesis of $PtdIns(4,5)P_2$ is initiated by the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate [PtdIns(4)P] by phosphatidylinositol 4-kinase (PtdIns 4-kinase). Although cells contain several PtdIns 4-kinases, the enzyme responsible for regulating the synthesis of hormone-sensitive $PtdIns(4,5)P_2$ pools has not been identified.

We observed that micromolar concentration of WT inhibit the synthesis of hormone-sensitive PtdIns(4)P and $PtdIns(4,5)P_2$ pools in intact adrenal glomerulosa cells, and demonstrated the presence of a WT-sensitive PtdIns 4-kinase in adrenocortical extracts. In addition to its sensitivity to the PtdIns 3-kinase inhibitor WT, this enzyme is distinguished from the recognized membrane-bound PtdIns 4-kinases by its molecular size and weak membrane association. Inhibition of this PtdIns 4-kinase by WT results in rapid

loss of the hormone-sensitive PtdIns(4,5)P_2 pool in angiotensin II-stimulated glomerulosa cells. Consequently, WT treatment inhibits the sustained but not the initial increases in inositol 1,4,5-trisphosphate and cytoplasmic [Ca^{2+}] in a variety of agonist-stimulated cells, including adrenal glomerulosa cells, NIH 3T3 fibroblasts, and Jurkat lymphoblasts. These results indicate that a specific WT-sensitive PtdIns 4-kinase is critical for the maintenance of the agonist-sensitive polyphosphoinositide pool in several cell types.

Ang II-induced signaling and growth responses in glomerulosa cells. Ang II and ACTH are the major hormonal regulators of adrenal function. Ang II controls the growth and differentiation of the zona glomerulose and stimulates aldosterone production, whereas ACTH controls glucocorticoid secretion by the zona fasciculata and also acts in the zona glomerulosa as a potent stimulus of aldosterone secretion. In several Ang II target tissues, AT_1 receptors interact with two distinct guanine nucleotide binding proteins, $G_{q/11}$ and G_i, to activate phospholipase C and to inhibit adenylate cyclase, respectively. However, in cultured bovine glomerulosa cells Ang II potentiates rather than inhibits the stimulatory effect of ACTH on cAMP levels. This effect of Ang II was partially mimicked by phorbol 12-myristate 13-acetate (PMA) and was partially inhibited by staurosporine or depletion of protein kinase C but was unaffected by pertussis toxin treatment. No potentiation was detectable in disrupted cells or in membrane preparations. In intact glomerulosa cells, treatment with cyclosporin A or FK506 completely inhibited Ang II or PMA-induced potentiation of cAMP production without affecting the response to ACTH. In COS-7 cells transfected with the rat AT_1 receptor, Ang II caused 2-3-fold enhancement of the ACTH-induced cAMP response, an effect that was partially reproduced by PMA. These potentiating actions of Ang II and PMA were prevented by preincubation with cyclosporin A or FK506, and the latter effect was abolished by rapamycin. These results implicate the Ca^{2+}- and calmodulin-dependent protein phosphatase, calcineurin, in Ang II-induced enhancement of adenylate cyclase activity in both adrenal glomerulosa and transfected COS-7 cells. The finding that Ang II enhances ACTH-stimulated production of cAMP by a second messenger-mediated mechanism that involves the participation of calcineurin reveals an additional mode of cross-talk between pathways activated by Ca^{2+}-mobilizing and cAMP-generating receptors.

The effects of Ang II on cell growth and proliferation have been extensively studied because of its potential role in cardiac and smooth muscle hypertrophy. Ang II is also a major factor in the hypertrophy and hyperplasia of adrenal cells that occur during dietary sodium deficiency. We studied the effects of Ang II on growth responses of primary cultures of bovine adrenal glomerulosa cells to explore the mechanism(s) by which Ang II leads to hyperplasia and hypertrophy of the glomerulosa layer in sodium deficiency. Ang II did not increase [^{3}H]thymidine incorporation during the first 5 days of culture, but mitogenic responses to Ang II became evident after longer periods of culture and were most prominent between 8 and 11 days after seeding. At this time, cell cycle analysis showed that Ang II increased the proportion of cells in the S phase and did not cause accumulation of cells in the G_2 phase. Consistent with the finding, Ang II also stimulated proliferation of glomerulosa cells during treatment for 3 days in the presence of 1% serum. The mitogenic effect of Ang II was not inhibited by pretreatment with pertussis toxin and was mediated by AT_1 receptors as indicated by its sensitivity to the subtype-selective antagonist DuP-753. Also, there was no emergence of AT_2 receptors in glomerulosa cells during prolonged culture. These results indicate that intracellular mechanisms that mediate growth responses become more active during prolonged culture of glomerulosa cells. Thus, in addition to regulating the steroidogenic and secretory functions of the zona glomerulosa, Ang II exerts mitogenic actions that depend on the functional state of the glomerulosa cells.

Significance to Biomedical Research and the Program of the Institute. Elucidation of the properties of angiotensin receptors and their mechanisms of target-cell activation will contribute to the understanding

of normal and disordered mechanisms of body fluid homeostasis and cardiovascular regulation. Ang II receptors are widely distributed throughout the vascular system, adrenal, kidney, and brain, and the clarification of their mechanisms of action at these sites will be facilitated by detailed analysis of the adrenal receptors and their signaling mechanisms. The importance of angiotensin II and its receptors in hypertension and cardiovascular disease has been demonstrated not only in animal models, but also by the therapeutic actions of angiotensin converting enzyme inhibitors and Ang II antagonists in essential hypertension and cardiac failure. Analysis of the structure-function properties of the Ang II receptor and its subtypes should provide new approaches to the design of Ang II antagonists, and to the understanding of mechanisms involved in the actions of Ang II on contractile, secretory, and growth responses in its numerous target tissues.

Proposed Course: The actions of Ang II on early signaling pathways and growth responses, and the structure-function properties of the Ang II receptor, will be further studied in adrenal glomerulosa cells, stably transfected Y_1 cells, and transiently transfected COS cells. The mechanisms by which the Ang II-induced calcium signal, in particular its sustained calcium influx phase, is generated and maintained during agonist stimulation will be investigated in glomerulosa cells and Y_1 cells, with emphasis on the role of $Ins(1,4,5)P_3$ in the influx process. The manner in which the early calcium signal promotes the expression of c-fos and other early response genes will also be studied in glomerulosa cells. Studies on the growth effects of Ang II in glomerulosa cells will address the activation of mitogenic responses that reflect the trophic action of Ang II on the zona glomerulosa *in vivo*, and the extent to which these are driven by Ang II itself or by other local growth factors in the adrenal gland. The relation between the antagonist and agonist binding sites of the AT_1 receptor will be further investigated by mutational analysis of the external and intra-membrane domains of the receptor. The structural features involved in G protein coupling and internalization will also be evaluated in mutant and native AT_1 receptors. The newly-identified PI 4-kinase that is responsible for maintaining the agonist-sensitive pool of phosphoinositides in several cell types will be purified from the adrenal cortex and its cDNA will be cloned to permit structure-function studies on the enzyme and analysis of factors that regulate its expression.

Publications:

Balla T, Catt KJ. Phosphoinositides and calcium signaling: new aspects and diverse functions in cell regulation. Trends Endocrinol Metab 1994;5:250-55.

Baukal AJ, Hunyady L, Catt KJ, Balla T. Evidence for participation of calcineurin in potentiation of agonist-stimulated cyclic AMP formation by the calcium-mobilizing hormone, angiotensin II. J Biol Chem 1994;269:24546-49.

Hunyady L, Bor M, Balla T, Catt KJ. A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the AT1 angiotensin receptor, J Biol Chem 270:16602-16609, 1995.

Hunyady L, Bor M, Balla T, Catt KJ. Critical role of a conserved intramembrane tyrosine residue in angiotensin II receptor activation. J Biol Chem 1995;270:702-05.

Hunyady L, Bor M, Balla T, Catt KJ. Identification of a cytoplasmic ser-thr-leu motif that determines agonist-induced internalization of the AT_1 angiotensin receptor, J Biol Chem 1994;269:31378-82.

Hunyady L, Baukal AJ, Balla T, Catt KJ. Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction, J Biol Chem 1994;269:24798-804.

Hunyady L, Tian Y, Sandberg K, Balla T, Catt KJ. Divergent conformational requirements for angiotensin II receptor internalization and signaling, Kidney International 1994;46:1496-98.

Ji H, Zheng W, Zheng V, Catt KJ, Sandberg K. Genetic transfer of a nonpeptide binding site to a previously unresponsive angiotensin receptor. Proc Natl Acad Sci USA (in press).

Nakanishi S, Catt KJ, Balla T. A novel wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids, Proc Natl Acad Sci USA 1995;92:5317-5321.

Tian Y, Balla T, Baukal A, Catt KJ. Growth responses to angiotensin II in bovine adrenal glomerulosa cells, Am J Physiol 1995;268:E135-44.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00194-07-ERRB

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Steroid Biosynthesis and Metabolism in the Mammalian Adrenal Cortex

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C.A. Strott	Head, SSR	ERRB, NICHD
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The biotransformation of steroids by conjugation to a highly charged sulfonate group is a functionally important process that results in a marked change in the physicochemical properties of these essentially hydrophobic compounds. This fact notwithstanding, the biological significance of steroid sulfonation is poorly understood. The transfer of a sulfonate group (SO3-) to an appropriate acceptor site is carried out by enzymes termed sulfotransferases. By altering steroid polarity, sulfonation increases water solubility and modifies protein binding, thereby influencing steroid transportability and acting as an intracellular trapping or storage mechanism. Furthermore, by serving to either initiate an event (steroid sulfonate is the active form) or terminate an event (steroid sulfonate is the inactive form), sulfonation can function to regulate hormonal responsivity. One of the most active tissues to sulfonate steroids is the adrenal cortex; furthermore, specific steroid sulfotransferases are differentially expressed in functionally distinct adrenocortical zones. To seek insight into the molecular mechanisms and biological consequences of steroid sulfonation, we are currently investigating steroid-specific sulfotransferases as well as enzymes responsible for the production of the universal sulfonate donor (PAPS). Projects include: 1) isolation, characterization, antibody production, and cDNA cloning and expression of specific enzymes; 2) genomic cloning and assessment of transcriptional regulation; 3) mutational studies to identify amino acid residues that create steroid substrate and sulfonate donor sites of interaction; 4) experiments regarding the structural basis for chiral specificity. The complete estrogen sulfotransferase (EST) gene, including the 5'-flanking region, has now been cloned and examination of its transcriptional regulation by steroid hormones is ongoing. An amino acid motif near the C-terminus of EST that is critical to binding of the sulfonate donor molecule has been identified and amino acid residues involved in estrogen interaction with EST have been specified by site-directed mutagenesis. The cDNA for chiral-specific 3b-hydroxysteroid sulfotransferase (HST) has been cloned and expressed, and structural genes for 3a- and 3b-HST have been partially cloned.

Project Description

Objectives:

The biotransformation of compounds by sulfonation occurs widely and markedly alters the physicochemical properties of the sulfonated products. Sulfonation of drugs and xenobiotics functions primarily to inactivate and clear these generally hydrophobic compounds, although there are examples where the active form of a drug is sulfonated. Likewise, the sulfonation of endogenous substances is a fundamental metabolic mechanism that covers an extended range of molecules of diverse size, structure, chemistry and function. Membrane and secretory proteins are post-translationally modified by sulfonation. Macromolecules such as glycosaminoglycans and proteoglycans, components of cell surface and connective tissue structures, are subjected to modification by the addition of sulfonate groups. Importantly, a multitude of low molecular weight compounds including neurotransmitters and hormones such as catecholamines, iodothyronines and steroids, are modified by sulfoconjugation.

Steroids constitute a unique subclass of lipids derived from activated five-carbon isoprene units. They are thus members of an extensive and diverse family of isoprenoids or terpenes which include, in addition to numerous isoprenoid polymers such as natural rubber, ubiquinone, dolichols, isopentenyl tRNA, prenyl derivatives, the fat soluble vitamins and cholesterol as well as its steroid and vitamin D derivatives. Steroids, which evolved before eukaryotes approximately two billion years ago, are ancient bioregulators representing one of the earliest forms of an information transmitter that exerted its message in the cell of origin. Steroids, in turn, are subject to various modifications including conjugation reactions. Of the latter, sulfoconjugation is qualitatively and quantitatively the most important to occur in extrahepatic tissues. It is particularly noteworthy that steroid sulfonation is a prominent reaction in steroidogenic tissues (adrenal, ovary, testis and placenta), although the biological import of this long-known fact has yet to be realized. Sulfonation, the transfer of a sulfonate group (SO_3^-) from an activated sulfonate donor to an appropriate steroid hydroxyl acceptor site, is carried out by enzymes termed sulfotransferases and leads to a marked change in the physicochemical property of steroids as a result of the insertion of the highly charged sulfonate moiety. With pKaS < 1.5, steroid sulfonates remain fully ionized at any pH encountered in a biological system.

Depending on the tissue and biochemical system, steroid sulfonation presents several physiological implications. For instance, by increasing the polarity of steroids, sulfonation increases water solubility and alters protein binding which can influence transportability and act as an intracellular trapping or storage mechanism. Steroid sulfonation can regulate steroid hormone responsivity. That is, steroid sulfonation can either initiate a physiological response (steroid sulfonate is the biologically active form) or it can terminate a response (steroid sulfonate is the inactive form); there is experimental evidence in support of both eventualities. It has also been demonstrated that steroid sulfonates can serve as substrates for P_{450} steroidogenic enzymes. These meaningful considerations notwithstanding, a full understanding of steroid sulfonation remains to be realized. Interestingly, one of the most active tissues to sulfonate steroids is the adrenal cortex; however, the reason for this remains a total mystery. Why steroid sulfotransferases should exist in the mammalian adrenal cortex and what precise biological role(s) these enzymes might play in this tissue remains to be determined. In keeping with our long-standing interest in steroid biosynthesis and metabolism, particularly as related to mammalian adrenal physiology, we have recently focused on the phenomenon of steroid sulfoconjugation in this tissue using the guinea pig adrenal, which resembles the human counterpart both structurally and biochemically, as an animal model. In order to develop an understanding of the process as well as the

biological significance of steroid sulfonation, it is first necessary that specific sulfotransferase enzymes be isolated, characterized, cloned and their transcriptional regulation and cell-specific expression be examined.

The primary function of the mammalian adrenal cortex is considered to be the production of a potent glucocorticoid (cortisol) and mineralocorticoid (aldosterone), steroid hormones that play indispensable roles in maintenance of the *internal milieu.* In a broader outlook, however, this view is perhaps too simplistic since it belies the complex structural and biochemical nature of this endocrine organ system. In fact, a large number and variety of steroids other than cortisol and aldosterone, including steroid sulfonates, are produced and secreted. It must be understood that the adrenal cortex (excluding such animals as rats and mice) is also capable of producing estrogenic and androgenic steroids, in addition to glucocorticoids and mineralocorticoids. The local as well as systemic influence of these potentially bioactive steroids is not comprehended. Estrogen and androgen receptors have been reported to exist in the mammalian adrenal cortex, although they have yet to be carefully examined. Structurally, the mammalian adrenal cortex is essentially composed of three concentric regions or zones of more or less distinct cell populations. The outermost zona glomerulosa is the site of aldosterone production and is primarily controlled by the renin-angiotensin system and potassium. The intermediate zona fasciculata produces cortisol and is principally controlled by the pituitary hormone adrenocorticotropin (ACTH). The innermost zona reticularis, although capable of producing a variety of steroid compounds, remains a perplexity in that its function is ill-defined and it lacks a definitive physiologic regulator. Thus, its *raison d'être* remains to be established.

We have made the intriguing observation that estrogen sulfotransferase (EST) and the stereoselective 3α- and 3β-hydroxysteroid sulfotransferases (3αHST, 3βHST) are differentially expressed in the functionally distinct zones of the adrenal cortex. The zona glomerulosa contains no detectable steroid sulfotransferase, the zona fasciculata contains only EST and the zona reticularis contains EST and both HSTs. In the guinea pig animal model, we have calculated that roughly 13% (undoubtedly an over-estimate) of the soluble protein in the zona reticularis consists of 3αHST, a truly remarkable level of abundance. Furthermore, the amount of 3αHST far exceeds that of 3βHST and EST. The predominance of 3αHST in the reticularis suggests a role of some significance for the enzyme in this cell type. EST is also abundant in the adrenal cortex, yet about two-thirds of the total adrenal estrone and estradiol is in the unconjugated form. This raises the question: does EST function solely as an estrogen sulfotransferase or might it have another role? Such a possibility is fostered by the finding that EST binds estradiol and, surprisingly, also pregnenolone with high affinity. This is coupled with the fact that the binding activities are dependent on a nucleotide cofactor. Moreover, the steroid binding activities are physically separated from the catalytic activity in that they are associated with distinct charge isoforms.

The meaning of restricting sulfonation of 3-hydroxylated steroids to the zona reticularis is obscure. Certainly, equivalent steroid substrates are present in the zona fasciculata as well as the zona reticularis. It is equally obscure why the zona glomerulosa should be devoid of steroid sulfotransferases. Such observations have produced a strong stimulus for us to seek an understanding of the molecular as well as physiological basis for the differential expression of these intriguing proteins in the adrenal cortex. It is anticipated that with the cloning of genes for various members of the steroid sulfotransferase family, significant progress towards this goal can be more directly achieved.

An important aspect of sulfonation has to do with the sulfonate donor. In mammals, the universal activated sulfonate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which is synthesized from

ATP and inorganic sulfate by the enzymes ATP sulfurylase and adenosine 5'-phosphosulfate kinase. The availability of PAPS, as well as the activity of sulfotransferase, could limit the rate of sulfonation, particularly when the sulfate ion concentration is in the physiological range. There is, however, little available data regarding the level of PAPS in various tissues of different species. Thus, we are interested in investigating PAPS formation in our animal model system and have embarked on a project to isolate, characterize, and clone PAPS synthase.

Major Findings:

Proposed active site domain in estrogen sulfotransferase. Inherently, enzymes that carry out the transfer of a sulfonate group must interact with two substrates, namely, the sulfonate donor and acceptor molecules. In mammals, the universal activated sulfonate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS). We recently published a report presenting evidence that a GxxGxxK sequence, related to the P-loop motif found in ATP- and GTP-binding proteins, is involved in the binding of PAPS by guinea pig estrogen sulfotransferase (gpEST). This motif is positioned within a highly conserved region located near the C-termini of all steroid and phenol sulfotransferases for which primary structures are known. To examine the GxxGxxK motif in greater detail, point mutations that replaced the conserved glycines (G260 and G263) and lysine (K266) with alanines were introduced singly or in combination into gpEST cDNA. Additionally, as "controls," non-motif residues S262, D264 and N267 were also mutated to alanine, singly or in combination.

The 9 mutant constructs generated from wild type gpEST cDNA were all found to express the 34 kDa gpEST protein (verified by immunoblot analysis). The expressed proteins were subjected to kinetic analysis to detect perturbations in the interaction with PAPS. Because K_m reflects a complex ratio of the individual rate constants, the measurement was used primarily to reflect changes in the overall catalytic efficiency of the expressed enzyme. No attempt has yet been made to determine whether ligand affinity or K_{CAT} or both might be affected by specific mutations. The expressed proteins were also examined for their ability to be photoaffinity labeled with [^{35}S]PAPS. While the mutation of a single motif residue had little effect on the interaction between gpEST and PAPS as determined by kinetic analysis as well as photoaffinity labeling, the mutation of any two residues in concert resulted in an approximate 10-fold increase in the K_m for PAPS and reduced photoafffinity labeling. The mutation of all three motif residues resulted in an inactive enzyme and complete loss of photoaffinity labeling, although the enzyme was expressed in a reasonable amount. On the other hand, the K_m for PAPS was unchanged from wild type for either double or triple "control" mutants, demonstrating the specific importance of the motif residues. Interestingly, several double motif mutants also displayed a striking change in K_m for the steroid substrate, again demonstrating greater perturbations (8- to 28-fold increase in K_m) than did single mutants; as noted above, the triple motif mutant was completely inactive. Unexpectedly, whereas the mutation of non-motif residues had a negligible effect on the K_m for PAPS, a marked increase in the K_m for the estrogen substrate (>30-fold) was noted.

In summary, data from mutational studies have indicated that G260, G263, and K266, amino acid residues that constitute the GxxGxxK P-loop motif, play a critical role in the interaction between guinea pig estrogen sulfotransferase and the universal sulfonate donor, PAPS. Additionally and importantly, residues G260 and N267 have profound effects on the ability of the enzyme to efficiently sulfonate the estrogen substrate. Based on these novel findings, it is proposed that the sequence GISGDWKN, encoded by the 5'-end of the 3' terminal exon of the gpEST gene, represents an essential component of the active site for gpEST. Also, that in so far as the GxxGxxK motif is universally conserved, this area will also prove to be catalytically important for all steroid and phenol sulfotransferases.

Structural gene for estrogen sulfotransferase and identification of a common splice site for steroid and phenol sulfotransferases. The gene for gpEST was found to be 18.6 kb in length, including the 3-kb 5'-flanking region that we previously cloned, and is composed of 8 exons and 7 introns. The splice junction donor/acceptor patterns were found to be in keeping with consensus sequences. Interestingly, it was noted that the 3'-terminal exon (#8) splice site involves the N-terminal glycine of the GxxGxxK P-loop motif. Interestingly, the 3'-terminal exon splice site of the 3α-HST gene we were concurrently cloning, along with five cloned steroid and phenol sulfotransferase genes reported in the literature, occurs at the codon for the same conserved glycine. Thus, based on the 3'-terminal exon splice site finding, the invariant conservation of the GxxGxxK motif and the similarly sized C-terminal protein fragment encoded by the 3'-terminal exons, we have proposed that all steroid and phenol sulfotransferase primary transcripts contain an identical splice site for the 3'-terminal exon that involves the upstream glycine of the GxxGxxK motif.

Analysis of the amino acid alignment for 19 reported cloned steroid and phenol sulfotransferases starting with the GxxGxxK motif revealed that each steroid and phenol sulfotransferase gene 3'-terminal exon will encode a similarly sized C-terminal fragment of the protein. Interestingly, on further analysis of the alignment, three distinct amino acid patterns emerge. The presence of the conserved functional GxxGxxK motif suggests that the protein domains encoded by steroid and phenol sulfotransferase 3'-terminal exons have evolved from a common ancestor. Furthermore, we hypothesize that during the course of evolution, the 3'-terminal exons further diverged into at least three sulfotransferase subdivisions: a phenol or aryl group, an estrogen or phenolic steroid group, and a neutral steroid group.

Intrinsic pregnenolone binding activity of estrogen sulfotransferase. As previously reported, our work on the guinea pig adrenocortical 34 kDa pregnenolone-binding protein (PBP) that had been purified to homogeneity and sequenced unexpectedly resulted in the cloning and expression of 34 kDa estrogen sulfotransferase (EST). We therefore assumed that the pregnenolone binding activity was a contaminant that co-purified with EST. Certainly, the structures of pregnenolone and estradiol are quite distinct. Nevertheless, the fact that the two activities did co-purify raised the question as to whether they might somehow be coupled. Additionally, there was the provocative finding that both pregnenolone and estradiol are bound with high affinity by guinea pig adrenocortical cytosol or partially purified protein. Furthermore, the binding activity for both steroids is abolished by treatment with alkaline phosphatase and is totally reversed by the addition of a heat stable factor that we identified as adenosine-3',5'-diphosphate (3',5'-ADP).

We determined that cytosol prepared from Chinese hamster ovary (CHO) K-1 cells transfected with gpEST cDNA demonstrates estrogen sulfotransferase activity, whereas cytosols prepared from nontransfected CHO-K1 cells and CHO-K1 cells transfected with vector without the gpEST cDNA insert are devoid of activity. It was further demonstrated that cytosol of CHO-K1 cells transfected with gpEST cDNA, in contrast to cytosol of nontransfected CHO-K1 cells, contains a high-affinity estradiol binding activity that is dependent on the presence of 3',5'-ADP, a finding similar to that made in partially purified guinea pig adrenocortical EST. These results indicated that the guinea pig adrenocortical estradiol binding activity was contained in EST. CHO-K1 cells transfected with gpEST cDNA offered an ideal situation in which to examine the issue as to whether or not EST and the pregnenolone binding activity are coupled. Thus, it was of considerable interest to find that cytosol prepared from CHO-K1 cells transfected with gpEST cDNA demonstrated saturable binding for pregnenolone as well as for estradiol. Importantly, the pregnenolone binding activity, as was the case for the estradiol binding activity, was dependent on the presence of 3',5'-ADP. Furthermore, pregnenolone and estradiol effectively competed with each other for binding, suggesting that the two

steroids bound to the same site. In contrast, pregnenolone was without effect on the sulfonation of estradiol, a result that is not easily understood. The steroid binding competition experiments clearly suggest that pregnenolone and estradiol bind to a common site on EST. On the other hand, the catalytic experiment suggests the presence of two distinct steroid interacting sites on EST, a catalytic site and a high affinity binding site. Notwithstanding the pregnenolone binding activity, expressed gpEST will not sulfonate pregnenolone. It should be noted that the binding activity of EST for pregnenolone is specific since steroids that are structurally closely related to pregnenolone, such as dehydroepiandrosterone and 17-hydroxypregnenolone, are not bound to EST. These data plus the results of related experiments support the conclusion that the steroid binding and catalytic activities of EST are physically separate, and are located on distinct charge isoforms. A more complete understanding of the biochemical and physiological significance of this interesting new finding must await additional experimentation on protein structure and definitive localization of the steroid binding pockets.

Neutral hydroxysteroid sulfotransferase cDNA. The cDNA for guinea pig 3β-hydroxysteroid sulfotransferase (3βHST) has been cloned and expressed in CHO-K1 cells. 3βHST is highly homologous to its chiral-specific counterpart, 3αHST. Whereas, 3αHST acts on neutral steroids where the 3-hydroxyl group is oriented in the alpha position, eg. androsterone and allopregnanolone, 3βHST acts on neutral steroids that have the 3-hydroxyl group oriented in the beta position, eg. pregnenolone and dehydroepiandrosterone. The two proteins consist of 287 amino acids, are 86% identical at the amino acid level and 88% identical at the nucleotide level. At 34,087 daltons 3βHST is slightly larger than 3αHST, which has a molecular weight of 33,637 daltons. 3αHST is a somewhat more acidic protein with a calculated pI of 6.2, whereas 3βHST which has a calculated pI of 6.7. 3βHST, as is the case for 3αHST, contains the PAPS-binding P-loop motif, GxxGxxK, near the C-terminus which is identically located for both proteins.

Significance to Biomedical Research and the Program of the Institute:

It is well established that steroid hormones secreted by the mammalian adrenal cortex play a crucial role in stress as well as immune responses. In addition, adrenal steroids can markedly influence reproduction and have a profound affect on normal growth and development. All of the aforementioned processes are subject to intense biomedical research and are of importance to the program of the Institute.

The adrenal cortex is the sole source of two potent steroid hormones, cortisol and aldosterone, that are essential for maintenance of the *internal milieu* and a normal healthy state. Cortisol, the principal glucocorticoid, is known to be secreted in increased amounts in response to stressful stimuli; however, its 'basal' biological function is not well understood. Aldosterone is the principal mineralocorticoid or salt-retaining steroid hormone. It is thus important to understand how the adrenal cortex responds to various effectors, and how the biosynthesis and metabolism of adrenocortical steroids are influenced and modulated. The adrenal cortex produces a large array of steroid compounds, some of which can be structurally and functionally classified as either glucocorticoids or mineralocorticoids, while others are structurally and functionally related to the sex steroids, estrogens and androgens. The biological significance of the local production of this wide range of bioactive steroids by the adrenal cortex is not presently understood. In addition, the adrenal cortex actively modifies steroids by the process of sulfonation and the biological importance of this fact is also not understood. The adrenal cortex is a complex endocrine organ system and the level of complexity is increased by the fact that it does not consist of a homogeneous population of cells, but rather multiple populations that are arranged into concentric regions or zones. While a considerable amount is known about the outermost zona glomerulosa (source of aldosterone) and the middle zona fasciculata (source of cortisol), it is rather

astonishing that, to date, the functional status of the innermost zona reticularis remains an enigma. In the human being, the zona reticularis remains essentially undeveloped until puberty, at which time it begins to form. Associated with this evolvement is a dramatic increase in the production and secretion of dehydroepiandrosterone and its sulfonated form, a steroid structurally related to androgens but without any known innate biological activity. The regulatory elements involved in this phenomenon are yet to be determined.

What about steroid sulfonates? Do they have an intrinsic biological function? In the central nervous system, pregnenolone sulfate and dehydroepiandrosterone sulfate have been shown to be potent antagonists of benzodiazepine-GABA$_A$ receptors that regulate chloride channels and thus are neuroexcitatory. In this regard, it is of interest that benzodiazepine receptors are also present in peripheral tissues, a particularly rich source being the adrenal cortex where they have been found to be located on outer mitochondrial membranes. This finding is potentially important because mitochondria are the site of the rate-limiting step in steroidogenesis, i.e. the conversion of cholesterol to pregnenolone. Steroid sulfonates are known to influence biochemical processes; for instance, estradiol sulfate but not free estradiol is a potent inhibitor of glutathione S-transferase. The sulfoconjugation of estrogenic steroids has emerged as an important mechanism for modifying these compounds. For example, in humans the most abundant circulating form of estrogen is estrone sulfate, which is present in 10- to 15-fold greater concentration in both men and women than the unconjugated form of either estrone or estradiol. It is important to note in this regard that estrone sulfate can be converted in target tissues to unconjugated estrone by an estrone sulfate-specific sulfatase, and the free estrone subsequently reduced to biologically active estradiol. Breast cancer cells have been found to derive a significant amount of unconjugated estrogen from extracellular estrone sulfate. Furthermore, in post-menopausal women, plasma estrone sulfate serves as the major source of estradiol available to breast cancer cells. On the other hand, the sulfoconjugation of estrogens in uterine and breast tissues can operate to regulate the level of biologically active free hormone. Interestingly, we recently reported that in the adrenal cortex a high concentration of EST immunoreactivity is present in nuclei, suggesting that EST might play a role in modulating the ability of active estrogens to regulate gene expression in ACTH-responsive cells.

As previously noted, steroid sulfotransferases are differentially expressed in the mammalian adrenal cortex. Although the mechanistic and biological bases for this intriguing cellular distribution are not understood, it seems likely that they must be related to the development of adrenocortical zonation along with the distinct functional status and steroidogenic activity associated with each zone. It is clear that, without an understanding of the *raison d'être* for the presence of steroid sulfotransferases in the mammalian adrenal cortex, a full understanding of this critical endocrine organ system will be lacking.

Proposed Course:

A. cDNA cloning of chiral-specific 3β-HST. This has just been completed and the results are being prepared for publication.

B. Genomic cloning of the chiral-specific 3α-HST. A partial clone covering a substantial 3' segment of the gene has been obtained, and cloning of the remaining portion plus the 5'-flanking region is ongoing.

C. Genomic cloning of the chiral-specific 3β-HST has now been initiated.

D. Isolation, characterization and sequencing of guinea pig PAPS synthase. This project is well advanced and should soon be completed.

E. cDNA cloning of guinea pig PAPS synthase.

F. cDNA cloning of human PAPS synthase. This has been initiated using an expressed sequence tag obtained from Craig Venter's data base.

G. Studies of EST transcriptional regulation.

H. Structural determinants for 3α-HST/3β-HST stereospecificity.

I. Studies of transcriptional regulation for the neutral hydroxysteroid sulfotransferases.

J. Isolation, characterization and cloning of testosterone sulfotransferase.

K. Isolation, characterization and cloning of cholesterol sulfotransferase.

Publications:

Chiba H, Komatsu K, Lee YC, Tomizuka T, Strott CA. The 3'-terminal exon of the family of steroid and phenol sulfotransferase genes is spliced at the N-terminal glycine of the universally conserved GxxGxxK motif that forms the sulfonate donor binding site, Proc Natl Acad Sci USA (in press).

Driscoll WJ, Komatsu K, Strott CA. Proposed active site domain in estrogen sulfotransferase as determined by mutational analysis, Proc Natl Acad Sci USA (in press).

Komatsu K, Driscoll WJ, Koh YC, Strott CA. A P-loop related motif highly conserved in sulfotransferases is required for binding the activated sulfate donor, Biochem Biophys Res Commun 1994;204:1178-1185.

Lee YC, Komatsu K, Driscoll WJ, Strott CA. Structural and functional characterization of estrogen sulfotransferase isoforms: distinct catalytic and high-affinity binding activities, Mol Endocrinol 1994;8:1627-1635.

Lee YC, Park C, Komatsu K, Kwack J, Strott CA. Adrenocortical pregnenolone binding activity resides with estrogen sulfotransferase, Endocrinology 1995;136:361-4.

Tomizuka T, Oeda T, Tamura Y, Yoshida S, Strott CA. Characterization of guinea pig estrogen sulfotransferase expressed by Chinese hamster ovary cell-K1 stable transfectants, Endocrinology 1994;135:938-943.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD00195-02 ERRB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intracellular Signaling in Endocrine Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

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Mathematical Research Branch, NIDDK (J. Rinzel)
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LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Unit on Cellular Signaling

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Coupling of GnRH and ET receptors with phospholipase C- and D-dependent intracellular signals was examined in pituitary cells. GnRH induced both initial and sustained IP3/DAG production, while ET-1 induced only a transient response, a profile that was comparable to the GnRH-induced DAG response in protein kinase C-depleted cells. GnRH- and ET-induced IP3/DAG and calcium responses were reduced by phospholipase C inhibitors, U73122 and neomycin. Those two phospholipase inhibitors also reduced agonist-induced LH release by cultured pituitary cells. In contrast to phospholipase C, phospholipase D activity was stimulated by agonist activation of GnRH but not ET receptors. Furthermore, GnRH- but not ET-induced DAG formation was reduced in the presence of the two phospholipase D inhibitors, ethanol and propanolol, and the fall in DAG production was accompanied by the inhibition of GnRH-induced c-fos expression. We have also examined the interactions between these receptor-mediated intracellular signals and plasma membrane electrical events. The results showed that pituitary cells are excitable and that each calcium spike is produced by the calcium entry during a single action potential (AP). The frequency of APs and calcium oscillations in gonadotrophs was modulated by depolarization/hyperpolarization pulses. Using these and other data on plasma membrane channel characterization in gonadotrophs provided earlier, a quantitative mathematical model predicting spatio-temporal calcium oscillations was developed. The model contains a set of Hodgkin-Huxley-like equations and the equation for calcium diffusion towards the cell center. It suggests that AP-induced calcium spiking is prominent only in a thin shell layer neighboring the cell surface. We also found that in GnRH-stimulated cells a brief depolarization pulse during the sustained phase can elicit a transient calcium rise similar to the endogenous cycle. In addition, calcium entry during a single depolarizing pulse was found to shift the phase of subsequent endogenous calcium oscillations, without affecting their frequency. The application of two consecutive depolarizing pulses showed that the size of the calcium rise evoked by the second pulse depended on the time lapsed between two consecutive pulses, indicating that each endogenous or evoked calcium rise cycle leaves the calcium release mechanism of the gonadotroph in a refractory state. Electrical activity may play an important role in the modulation of phospholipase C-induced calcium oscillations, including resetting the clock of the oscillator.

Project Description

Objectives:

To clarify the cellular signaling cascade in endocrine and neuroendocrine cells operated by calcium-mobilizing receptors, and the interactions between plasma membrane electrical events and receptor-mediated signaling. In particular, to characterize the spatial and temporal patterns of phospholipase C- and phospholipase D-dependent intracellular signaling, and effects of the interactions between signaling molecules triggered by these two pathways on secretion and early response gene expression. The research includes studies on the electrophysiological characterization of spontaneous and agonist-induced plasma membrane and endoplasmic reticulum membrane excitabilities, the patterns and mechanisms of calcium oscillations, and the effect of inositol 1,4,5-trisphosphate ($InsP_3$)-controlled cytoplasmic calcium oscillations on membrane potential (V_m).

Methods Employed:

Dispersion and culture of pituitary and Leydig cells. αT3-1, GT1-7, and PT-r cell line cultures. Photometry and imaging techniques for measurements of cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) in single cells. Whole cell patch clamp studies in pituitary gonadotrophs. Inositol phosphate, diacylglycerol (DAG), cytidine diphosphate diacylglycerol (CDP-DAG), phosphatydylethanol (PEt), and phosphatidic acid (PA) measurements. RNA and Northern blot analysis of c-fos, c-jun, and junB in pituitary and αT3-1 cells. Radiolabeled ligand binding studies. Mathematical description of mechanisms and modeling of the data.

Major Findings:

Phospholipase C dependence of GnRH and endothelin actions in gonadotrophs. Pituitary gonadotrophs express at least two types of G protein-coupled calcium-mobilizing receptors, for gonadotropin-releasing hormone (GnRH-R) and endothelin (ET_A-R). Their activation initiates the phospholipase C/calcium signaling pathway that leads to gonadotropin secretion and gene transcription. The activation of phospholipase C by GnRH is a G protein-dependent process and has been shown to be mediated by pertussis toxin-insensitive G proteins, specifically G_q and/or G_{11}. GnRH induces rapid and prominent phosphoinositide turnover in pituitary cells, primarily by stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). In cultured pituitary cells and αT3-1 gonadotrophs, GnRH causes a rapid initial increase in the formation of $InsP_3$ and its metabolites, followed by a slower secondary elevation. In contrast, ET-1 induces only a monophasic $InsP_3$ response in cultured pituitary cells. However, conflicting evidence for the activation of phospholipase C during GnRH action has been reported in experiments utilizing U73122, an inhibitor of agonist-stimulated phospholipid hydrolysis. These data question the role of phosphoinositide hydrolysis in GnRH-induced $[Ca^{2+}]_i$ and secretory responses in gonadotrophs, and instead suggest the importance of GnRH-controlled Ca^{2+} entry and of GnRH-R coupling to multiple G proteins. This proposal implies that gonadotrophs differ from other cells in exhibiting agonist-induced kinetics of inositol phosphate formation that differ from their Ca^{2+} signaling kinetics.

To address this issue, the coupling of GnRH-R and ET-R to phospholipase C, and the dependence of LH secretion on phosphoinositide signaling pathways, were analyzed in pituitary cells treated with two inhibitors of phospholipid breakdown, U73122 and neomycin. The results in normal cells and

immortalized gonadotrophs showed that $InsP_3$ and DAG formation induced by GnRH (100 nM) and ET-1 (100 nM) was reduced by U73122 in a concentration-dependent manner, with IC_{50}s of ~2 µM and complete inhibition at 10 µM U73122. Neomycin also reduced GnRH- and ET-induced inositol phosphate production in both cell types. Agonist-induced $[Ca^{2+}]_i$ responses were also inhibited in both cell types by U73122 and neomycin, at the same concentrations that inhibited their inositol phosphate responses. Not only Ca^{2+} mobilization, but also agonist-induced Ca^{2+} entry was blocked, indicating that neither GnRH nor ET can activate influx of Ca^{2+} independently of $InsP_3$-induced Ca^{2+} mobilization. Thus, the activation of Ca^{2+} entry is tightly connected with the $InsP_3$-induced depletion of agonist-sensitive Ca^{2+} pool(s).

In addition, agonist-induced luteinizing hormone (LH) release by cultured pituitary cells was inhibited by U73122 and neomycin in a dose-dependent manner. In perifused pituitary cells, U73122 completely inhibited GnRH- and ET-1-induced LH release but after 10 min caused a progressive and substantial increase in basal LH release. In static cultures, U73122 inhibited agonist-induced LH response at low concentrations (up to 3 µM) but stimulated LH release at higher concentrations due to direct activation of exocytosis by the compound. When added alone, U731222 caused a concentration-dependent increase of LH release with EC_{50} of ~7 µM and a similar maximum response as elicited by GnRH. The stimulatory action of U73122 on LH release was not reduced in the absence of extracellular Ca^{2+}. In contrast to cultured pituitary cells, αT3-1 gonadotrophs showed only constitutive exocytosis that was not affected by either neomycin or U73122. These results demonstrate that GnRH and ET_A receptors are coupled to the phosphoinositide/Ca^{2+} transduction system in pituitary gonadotrophs, and provide evidence for the dependence of agonist-regulated exocytosis on this signaling pathway. The ability of U73122 to stimulate LH release could reflect an additional action of the compound at late steps in the exocytotic pathway.

Dependence of stimulus-transcription coupling on phospholipase D pathway. In addition to phospholipase C, activation of phospholipase D is commonly observed in cells activated by plasma-membrane tyrosine kinase and G protein-coupled Ca^{2+} mobilizing receptors. The PA formed during hydrolysis of phosphatidylcholine (PC) by phospholipase D, and the DAG produced during phospholipase C-mediated hydrolysis of PIP2, are interconverted by the actions of DAG-kinase and PA-phosphohydrolase. These products of receptor-stimulated phospholipase activity can be further metabolized through the CDP-DAG phosphoinositide cycle, or the lysophosphatidic acid (LPA) and CDP-choline pathways. In the presence of ethanol, phospholipase D catalyses a transphosphatidylation mechanism that leads to the formation of PEt, and this reaction is commonly used as a specific assay for phospholipase D activity in agonist-stimulated cells. In contrast to these well defined aspects of PC metabolism, the cellular functions of phospholipase D are still uncertain and the products of its activation that act as intracellular messengers remain speculative. DAG, PA, and LPA have been proposed to act as mediators in phospholipase D-dependent vesicular traffic, activation of phosphatidylinositol-4-phosphate kinase, stimulation of Ca^{2+} entry and exocytosis, early response gene expression, and mitogenesis. The fact that these cellular functions are also controlled by products of the phospholipase C pathway has complicated the analysis of the role of phospholipase D in their regulation.

We have examined the role of phospholipase D-derived signaling molecules in stimulus-transcription and -secretion coupling in anterior pituitary cells and immortalized αT3-1 gonadotrophs. In these cells, GnRH action was associated with both initial and sustained DAG production, while ET-1 induced only a transient DAG response, a profile that was comparable to the GnRH-induced DAG response in protein kinase C-depleted αT3-1 gonadotrophs. Also, phospholipase D activity, estimated by the production

of PEt from phosphatidylcholine in the presence of ethanol, was stimulated by GnRH but not by ET-1. The GnRH-induced PEt response was time- and concentration-dependent. Thus, phospholipase D activity is stimulated by agonist activation of the GnRH but not the ET_A receptors. Furthermore, such formation of PEt at the expense of PA during GnRH-induced activation of phospholipase D significantly reduced the production of not only PA, but also DAG and CDP-DAG. Inhibition of PA-phosphohydrolase activity by propranolol also decreased GnRH-induced DAG production and, in contrast to ethanol, increased PA and CDP-DAG levels. These data demonstrate that ethanol and propranolol can be used not only to evaluate the status of phospholipase D activity, but also to modify PA, DAG, and CDP-DAG levels in GnRH-stimulated cells.

Based on these observations, the effects of such maneuvers on GnRH- and ET-1-induced c-fos expression and gonadotropin release were examined. The fall in DAG production caused by ethanol and propranolol was accompanied by inhibition of GnRH-induced c-fos expression in a concentration-dependent manner. However, neither treatment affected agonist-induced LH release in static cultures during 3-h stimulation, nor the GnRH-induced secretory response in perifused pituitary cells. Thus, the signaling molecules generated by phospholipase D participate in stimulus-transcription coupling, but are not essential for agonist-induced exocytosis in pituitary gonadotrophs. Consistent with the lack of coupling of ET_A receptors to phospholipase D, neither ethanol nor propranolol affected ET-1-induced c-fos expression. The GnRH- and ET-1-induced inositol trisphosphate/calcium signaling was also not affected by ethanol and propranolol, further suggesting that these compounds specifically affect the phospholipase D pathway. These findings demonstrate that phospholipase D participates in stimulus-transcription but not stimulus-secretion coupling, and indicate that DAG is the primary signal for this action.

Plasma membrane excitability and Ca^{2+} signaling in gonadotrophs. Secretory cells in the anterior pituitary gland are electrically excitable. Spontaneous action potentials (APs) have been recorded in several cell types of normal pituitary cells, including lactotrophs, somatotrophs, melanotrophs, and also some pituitary cell lines. Calcium imaging and photometric experiments in unstimulated pituitary lactotrophs, somatotrophs, gonadotrophs, and GH cell lines also revealed spiking fluctuations in $[Ca^{2+}]_i$ around the basal level of 100-220 nM. The sensitivity of these APs and $[Ca^{2+}]_i$ fluctuations to extracellular Ca^{2+} concentrations and to calcium channel blockers indicate that these oscillations are produced by Ca^{2+} entry through voltage-sensitive calcium channels (VSCC). The present work (done in collaboration with Drs. Yue-Xian Li and John Rinzel, Mathematical Research Branch, NIDDK) further explores the quantitative relationship between voltage firing and the associated $[Ca^{2+}]_i$ spiking in unstimulated pituitary cells.

To address how the basal $[Ca^{2+}]_i$ fluctuations depend on changes in V_m, simultaneous measurements of V_m and $[Ca^{2+}]_i$ were performed in rat pituitary gonadotrophs. The data showed that each Ca^{2+} spike is produced by the Ca^{2+} entry during a single AP. Using these and the data on plasma membrane channel characterization in gonadotrophs, a quantitative mathematical model of this plasma membrane oscillator was developed. The model predicts not only temporal but also spatial distribution of $[Ca^{2+}]_i$. Such a distribution is obtained by solving Hodgkin-Huxley-like set of equations together with the equation for diffusion of Ca^{2+} towards the cell center. Since our focus was on the plasma membrane oscillator of unstimulated cells, the model assumption is that of the passive cytoplasm, i.e, it allows neither agonist-induced Ca^{2+} oscillations nor Ca^{2+}-induced Ca^{2+}-release, but is capable of the maintenance of a fixed Ca^{2+} level.

The model demonstrates that AP-induced $[Ca^{2+}]_i$ spiking is prominent only in a thin shell layer

neighboring the cell surface. This localized $[Ca^{2+}]_i$ spike transiently activates the Ca^{2+}-dependent potassium current, resulting in a sharp afterhyperpolarization following each voltage spike. In accord with experimental observations, the model shows that the frequency and amplitude of the voltage spikes are highly sensitive to current injection and to blocking of the Ca^{2+}-sensitive current. Computations also predict that, leaving the membrane channel intact, the firing rate can be modified by changing the Ca^{2+} handling parameters: Ca^{2+} diffusion rate, Ca^{2+} buffering capacity, and the rate of plasma membrane Ca^{2+} pump. The model provides a basis for further investigation on the role of plasma membrane electrical activity in the control of $[Ca^{2+}]_i$ in quiescent and agonist-stimulated cells.

Another important aspect of the Ca^{2+} entry pathway(s) in cells operated by Ca^{2+} mobilizing receptors is the dependence of agonist/$InsP_3$-induced $[Ca^{2+}]_i$ oscillations on V_m. We have previously analyzed the influence of V_m on $[Ca^{2+}]_i$ oscillations during the sustained extracellular Ca^{2+}-dependent phase of the calcium signaling response to GnRH using $I_{K(Ca)}$ as an indicator of Ca^{2+} oscillations. The results of these investigations indicated that in agonist- and $InsP_3$-stimulated cells, sustained $[Ca^{2+}]_i$ oscillations were extinguished by hyperpolarization after 3-15 min despite the availability of Ca^{2+} in the extracellular medium. Single depolarizing pulses transiently restored the amplitude of the sustained spiking in a dihydropyridine- and extracellular calcium-sensitive manner. The response to depolarization showed a marked dependence on V_m that was correlated with the establishment of steady-state inward calcium current.

In additional experiments, we clarified that in GnRH-stimulated cells a brief depolarization pulse can elicit a transient $[Ca^{2+}]_i$ rise similar to the endogenous cycle. Furthermore, Ca^{2+} entry during single depolarizing pulses was found to shift the phase of subsequent endogenous $[Ca^{2+}]_i$ oscillations, without affecting their frequency. Application of two consecutive depolarizing pulses showed that the size of the $[Ca^{2+}]_i$ rise evoked by the second pulse depended on the time elapsed between the two pulses, indicating that each endogenous or evoked $[Ca^{2+}]_i$ rise cycle leaves the Ca^{2+} release mechanism of the gonadotroph in a refractory state. Based on these observations, we proposed that the underlying mechanism in such refractoriness involves modulation of the $InsP_3$ receptor channel at the endoplasmic reticulum membrane.

Ca^{2+} excitability of the endoplasmic reticulum membrane. In collaboration with Drs. Yue-Xian Li and John Rinzel, Mathematical Research Branch, NIDDK and Dr. Joel Keizer, Institute of Theoretical Dynamics and Department of Chemistry, University of California, Davis, a further development on the mathematical model was done to describe several aspects of agonist-induced Ca^{2+} signaling in single gonadotrophs. The model was based on experimental observations in pituitary gonadotrophs, as well as in other cell types, that a small increase in $[Ca^{2+}]_i$ is able to trigger a series of large amplitude $[Ca^{2+}]_i$ spikes. This dynamic property is associated with the endoplasmic reticulum (ER) membrane and manifests itself only when $InsP_3$ is above its basal level, or at basal $InsP_3$ when the SERCA pump activity is reduced or the passive ER calcium leak is enhanced. Such Ca^{2+} excitability of the ER membrane is comparable to the electrical excitability of the plasma membrane as discussed above: i) the excitation is driven by potential energy stored in the form of trans-membrane concentration gradients of nonpermeable ions; ii) the excitation consumes energy, prestored in this gradient, which is reestablished through ATP-dependent active transport; iii) the excitation variable (either plasma membrane voltage or $[Ca^{2+}]_i$) controls the changes in membrane permeability.

Calcium signaling in single rat Leydig cells. The production of testicular androgens by the Leydig cells is controlled by endocrine interactions between the hypothalamus, pituitary and testis, as well as through paracrine and autocrine regulation within the testis. The predominant endocrine control of Leydig cell function is exerted through the LH receptor, which is coupled to the adenylate cyclase

pathway. Like several other adenylate cyclase-coupled receptors, the LH receptor is also capable of activating the inositol phosphate/calcium signaling pathway, probably through G_q or a related guanyl nucleotide regulatory protein. This response to activation of the LH receptor is well developed in ovarian granulosa cells and in heterologous cell types expressing LH receptors, but has not been consistently observed in the Leydig cell. In addition to gonadotropins, Leydig cell function is also influenced by growth factors and neuropeptides, including insulin-like growth factor I, transforming growth factor-α and -β, epidermal growth factor, GnRH, growth hormone-releasing hormone, corticotropin-releasing factor (CRF), vasopressin, oxytocin, angiotensin II, and ET. Most of these factors and hormones are produced locally within the testis and appear to exert paracrine or autocrine actions in the interstitial or tubule compartments. Several of these agonists operate through Ca^{2+}-mobilizing receptors in other cell types, further indicating that Ca^{2+} signaling may contribute to the control of Leydig cell function.

Although both gonadotropin- and GnRH-induced inositol phosphate/Ca^{2+} signaling have been reported in Leydig cells, the results of these investigations were incomplete and contradictory. To resolve these inconsistencies, the pattern of agonist-induced Ca^{2+} signaling was analyzed in individual Leydig cells stimulated with LH, hCG, GnRH, ET-1, CRF, and serotonin. These experiments were performed in collaboration with Dr. Maria Dufau, Endocrinology and Reproduction Research Branch, NICHD. The basal level of $[Ca^{2+}]_i$ in adult rat Leydig cells was 70 to 160 nM and was unaffected by high K^+ or the dihydropyridine calcium channel agonist, Bay K 8644. These findings are consistent with the absence of VSCC in the Leydig cells. In addition, no increase in $[Ca^{2+}]_i$ was observed in cells treated with LH, corticotropin-releasing factor, and serotonin. However, both GnRH and ET-1 induced rapid and transient elevation of $[Ca^{2+}]_i$ that was not associated with a sustained plateau phase, and was unaffected by removal of Ca^{2+} from the incubation medium. The amplitude of the $[Ca^{2+}]_i$ response was not dependent on the concentration of GnRH and ET-1, but the number of responsive cells increased with increasing agonist concentration with a maximum of about 30% of the Leydig cell population. The calcium-mobilizing actions of GnRH and ET-1 were abolished by the GnRH and ET_A receptor antagonists. The majority of the cells expressed solely GnRH or ET_A receptors, and about 10% expressed both receptors. GnRH-induced Ca^{2+} responses were observed almost exclusively in medium-sized Leydig cells, while ET-induced responses were most frequent in large Leydig cells. These data demonstrate that single Leydig cells expressing GnRH and ET_A receptors exhibit monophasic $[Ca^{2+}]_i$ responses that are activated in an all-or-none fashion. Such transient Ca^{2+} signaling may trigger short-term cellular responses, or could modulate the actions of gonadotropins acting through the cAMP signaling pathway.

Endothelins and Ca^{2+} signaling and secretion in parathyroid cells. Cytosolic Ca^{2+} is regarded as a primary regulator of the parathyroid hormone (PTH) secretion, but in contrast to other cell types that secrete by exocytotic mechanism, an increase in $[Ca^{2+}]_i$ immediately results in inhibition of PTH release. $[Ca^{2+}]$i in parathyroid cells is controlled by a Ca2+ receptor, i.e., by extracellular Ca^{2+} concentrations. The receptor operates in the range of 0.1 to 3 mM Ca2+ and changes [Ca2+]i from 20 to 500 nM with an EC50 of 1.2 mM. Over the same dose-range, $[Ca^{2+}]$e inhibits PTH secretion from bovine parathyroid cells with an IC50 of 1 mM. In this regard, the observation that Ca^{2+}-mobilizing agonist, ET-1, stimulates PTH release in a dose-dependent manner and without affecting the IC50 for $[Ca^{2+}]$e-dependent inhibition implies that secretion is controlled in calcium-independent manner, or $[Ca^{2+}]_i$ is differentially regulated by ETs differently in these cells.

To address this problem, we examined the effects of endothelins on $[Ca^{2+}]_i$ signaling and secretion in rat parathyroid cells. These experiments were done in collaboration with Dr. Kazushige Sakaguchi,

Bone Research Branch, NIDR. Consistent with the presence of an operative intracellular mechanism for Ca^{2+} release, the results showed that these cells express InsP$_3$ receptors and that ionomycin induced an increase in [Ca^{2+}]i in cell bathed in Ca^{2+}-deficient medium. Also, an RNA-polymerase chain reaction analysis revealed the expression of both ET$_A$ and ET$_B$ receptors in PT-r cells. Activation of these receptors by ET-1 was associated with a transient increase in [Ca2+]$_i$. Responsive PT-r cells can be divided into three groups according to the late sustained plateau level relative to the prestimulatory basal level: the plateau level is higher than the basal level, the level quickly returns to the basal level after the initial spike, and the plateau level is lower than the basal level. All three types of response were also observed in Ca^{2+}-deficient medium or in the presence of non-selective Ca^{2+} channel blockers. Furthermore, the percentage of the cells showing the plateau level below the initial increased with increasing in ET concentrations, suggesting that exclusion rather than entry is facilitated by activation of ET$_A$ receptors. Since the secretory response was measured 60 min after stimulation with ET-1, it is possible that the integrated effects of [Ca^{2+}]$_i$ rather than the kinetic profiles are essential for the secretory output. In this regard, the ability of ET-1 to maintain suppression of [Ca^{2+}]$_i$ for a prolonged period of time after the transient stimulation is consistent with its effects on hormone release.

Significance of Biomedical Research and the Program of the Institute:

The clarification of receptor-mediated intracellular signaling is essential for the understanding of both normal and disordered cellular functions. Current emphasis is placed on the characterization of Ca^{2+} signaling and Ca^{2+}-controlled cellular functions. In general, Ca^{2+} is involved in the activation of a complex set of intracellular enzymes, gene expression, hormone and neurotransmitter release, cell division and differentiation. Furthermore, cytosolic Ca^{2+} participates in intracellulular "crosstalk", i.e., is involved in activation of the other intracellular signaling systems such as the phospholipase D and phospholipase A$_2$ signaling pathways. The complex pattern of interactions between Ca^{2+} and protein kinase C signaling in controlling cellular functions, including the expression of early response genes and hormone secretion, further indicates the need for a comparative analysis of intracellular signaling in agonist-stimulated cells. Thus, the regulation of calcium mobilization from intracellular store(s), and calcium entry through a variety of plasma-membrane calcium channels is of central importance in the cellular physiology of endocrine and neuroendocrine cells.

Proposed Course:

Future studies will include more detailed characterization of calcium signaling in a variety of neuroendocrine and endocrine cells. Specific aspects of this program are: the temporal and spatial analyses of calcium signaling in agonist-activated target cells by confocal microscopy, including characterizing the "pace-maker" activity of a single cell; the speed of calcium signaling in oscillating and non-oscillating cells; the dependence of the propagation of calcium signaling on the structure of endoplasmic reticulum; and the distribution of plasma-membrane channels. Characterization of the amplification and attenuation of calcium signaling and research on the importance of voltage-insensitive and voltage-sensitive plasma membrane calcium channels in these processes will also be performed. In addition, the role of intercellular calcium signaling and electrical coupling as possible mechanisms in the communication between lactotroph and gonadotroph populations of pituitary cells will be analyzed.

To address the dependence of the interactions between InsP$_3$ receptor channels and plasma-membrane voltage-sensitive and -insensitive calcium channels on the level of depletion of the agonist-sensitive calcium pool, single cell imaging and confocal microscope analyses of sublocal calcium distribution

within the cells will be done. In addition, the integration of the plasma-membrane calcium channels into the operation of the $InsP_3$-controlled cytoplasmic oscillator will be further investigated by a combination of single photometry calcium measurements and whole cell patch clamp analysis. The investigations will include the characterization of the bidirectional effects of depolarization on $InsP_3$-induced Ca^{2+} spiking as the possible mechanism for the resetting of $InsP_3$-dependent calcium oscillations by depolarization in oscillatory cells.

We also plan to analyze the mechanism of phospholipase D activation in agonist-stimulated cells. Specific attention will be given to the possible role of Ca^{2+} entry through VSCC in the activation of phospholipase D in unstimulated and agonist-stimulated cells. For this purpose, studies will be done on electrically active endocrine and neuroendocrine cells in which this pathway is operative, and assays of DAG kinase, CDP-DAG, and PEt, will be performed. Also, the physiological significance of the possible integration of the phospholipase D pathway into the sequence of cellular events controlled by electrical activity will be addressed.

Publications:

Cesnjaj M, Zheng L, Catt KJ, Stojilkovic SS. Dependence of stimulus-transcription coupling on phospholipase D in agonist-stimulated pituitary cells. Mol Biol Cell 1995;6:1037-1047.

Fujii Y, Tomic M, Stojilkovic SS, Iida T, Brandi ML, Ogino Y, Sakaguchi K. Effects of endothelin-1 on Ca^{2+} signaling and secretion in parathyroid cells. J Bone Mineral Res 1995;10:716-725.

Jobin RM, Tomic M, Zheng L, Stojilkovic SS, Catt KJ. GnRH-induced potentiation of calcium-dependent exocytosis in pituitary gonadotrophs. Endocrinology 1995;136:3398-3405.

Keizer J, Li Y-X, Stojilkovic SS, Rinzel J. $InsP_3$-induced Ca^{2+} excitability of the endoplasmic reticulum. Mol Biol Cell 1995;6:945-951.

Li Y-X, Keizer J, Stojilkovic SS, Rinzel J. Calcium excitability of the ER membrane: an explanation for IP_3-induced Ca^{2+} oscillations. Am J Physiol 1995 (in press).

Li Y-X, Rinzel J, Vergara L, Stojilkovic SS. Spontaneous electrical and calcium oscillations in pituitary gonadotrophs. Biophys J 1995;69:785-795.

Olson B, Scott DC, Wetsel WC, Elliot SJ, Tomic M, Stojilkovic SS, Nieman LK, Wray S. Effects of insulin-like growth factors (IGF-I and IGF-II) and insulin on the immortalized hypothalamic GT1-1 cell lines. Neuroendocrinology 1995;62:155-165.

Spergel DJ, Krsmanovic LZ, Stojilkovic SS, Catt KJ. L-type Ca^{2+} channels mediate join modulation by GABA and glutamate of $[Ca^{2+}]_i$ and neuropeptide secretion in immortalized GnRH neurons. Neuroendocrinology 1995;61:499-508.

Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995 (in press).

Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995 (in press).

Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of GnRH receptors. Rec Prog Horm Res 1995;50:161-205.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second International Symposium on Fertility Regulation 1995 (in press).

Tomic M, Dufau ML, Catt KJ, Stojilkovic SS. Calcium signaling in single rat Leydig cells. Endocrinology 1995;136:3422-3429.

Vergara L, Stojilkovic SS, Rojas E. GnRH induced cytosolic calcium oscillations in pituitary gonadotrophs: phase resetting by membrane depolarization. Biophys J 1995 (in press).

Zheng L, Paik W-Y, Cesnjaj M, Balla T, Tomic M, Catt KJ, Stojilkovic SS. Effects of the phospholipase-C inhibitor, U73122, on signaling and secretion in pituitary gonadotrophs. Endocrinology 1995;136:1079-1088.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Human Biochemical Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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T. Isogai, Ph.D., Special Volunteer, HDB, NICHD
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P. Ye, Ph.D., IRTA Fellow, HDB, NICHD

COOPERATING UNITS *(if any)*

See Attached

LAB/BRANCH
Heritable Disorders Branch

SECTION
Section on Human Biochemical Genetics

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.3	6.3	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Members of the Section have linked the cystinosis gene to marker D17S1584 on the short arm of chromosome 17 with a maximum lod score of 10.89 at theta = 0.03. They have constructed a YAC contig spanning the region of interest in an attempt to isolate the cystinosis gene itself.
2. Several mutations in the Menkes disease gene have been identified in patients with this X-linked disorder, and genotypes have been correlated with responsiveness to early copper histidine therapy in two families. In one, a G to T transversion at a -1 exonic splice donor site caused a glutamine to histidine substitution at codon 724, with skipping of one, two, or three exons, and premature termination. Two related children with this mutation were treated early in life with copper histidine, and neither had a normal neurodevelopmental outcome, suggesting that the Q724H mutation did not preserve sufficient residual Menkes ATPase activity for significant clinical benefit to accrue from copper replacement. In contrast, a different family exhibited a 5-base duplication at a splice acceptor site just upstream of a 226 bp exon in the middle of the Menkes coding sequence, with one transcript which contained a deletion of the preceeding 77 bp exon. This created an open reading frame, and RNAse protection studies revealed 25% of the normal Menkes gene transcript. One affected boy in this family, treated with copper from 8 days of age, now has normal neurodevelopment at 14 months of age.
3. Analysis of N-linked oligosaccharides on serum glycoproteins from patients with Carbohydrate Deficient Glycoprotein Syndrome revealed heterogeneity in the biochemical basis of the disease, with some patients demonstrating a defect early in the formation of dolichol-oligosaccharides.
4. A tyrosine transport system has been characterized in the melanosomes of murine melanocytes and found to be functional in the pink-eyed dilution mutant, the murine homologue of oculocutaneous albinism type II.
5. Dolichols have been identified as components of the ceroid lipofuscin which is stored in the cellular lysosomes of patients with Hermansky-Pudlak syndrome, a type of albinism with a platelet storage pool defect.

Cooperating Units:

F. Tietze, Laboratory of Molecular and Cellular Biology, NIDDK
J. Schneider, University of California at San Diego
J. Thoene, University of Michigan
M. Kaiser-Kupfer, Clinical Branch, NEI
K. Ishak, Laboratory of Hepatic Pathology, AFIP
L. Charnas, Neurological Associates, Grand Junction, Colorado
L. Racusen, Johns Hopkins Hospital
D. Goldstein, Clinical Neuroscience Branch, NINDS
A. Mohan, George Washington University Medical Center
W. van't Hoff, Guy's Hospital, London
B. Brantly, NHLBI
E. Kvittingen, University of Oslo, Norway
M. Grompe, Oregon Center for Health Sciences, Portland
M. Polymeropoulos, NCHGR
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A. Tangerman, St. Radboud University Hospital, Nijmegen, Netherlands
C. Waechter, University of Kentucky College of Medicine, Lexington, KY
R. Miller, Bethesda Naval Hospital
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S. Tanner, Sheffield University, Sheffield, England
V. Hearing, National Cancer Institute
R. Boissy, University of Cincinnati
D. Theodoropoulos, Interinstitute Genetics Program, NIH Clinical Center
F. Skovby, Rigshospitalet, Copenhagen, Denmark
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W. Rhead, University of Iowa
N. Buist, University of Oregon
H. Fales, NHLBI
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T. Shawker, Dept. of Radiology, NIH Clinical Center
J. Ingelfinger, Massachusetts General Hospital, Boston, MA
M. Brilliant, Fox Chase Cancer Research Center, Philadelphia, PA
H. Andersson, Tulane University Medical Center, New Orleans, LA
L. Kohn, NIDDK

Project Description

Objectives:

The goals of this project are to: 1) isolate the cystinosis gene; 2) provide diagnosis and therapy for the renal and nonrenal complications of nephropathic cystinosis; 3) perform mutational analysis and phenotype/genotype correlation for Menkes disease patients; 4) perform mutational analysis on the Wilson disease gene in Indian Childhood Cirrhosis fibroblasts; 5) characterize new and rare metabolic disorders in man, such as Carbohydrate-Deficient Glycoprotein Syndrome, by describing patients clinically and determining the basic defect involved; 6) describe defects of lysosomal membrane transport and other new lysosomal storage diseases based on characterization of the stored material; 7) develop new methodologies for the analysis of carbohydrates in the urine of patients with rare disorders of carbohydrate metabolism; 8) determine the characteristics of tyrosine transport across normal and pink-eyed dilution murine melanosomal membranes; 9) identify the rab proteins expressed in cultured human melanocytes; 10) identify the stored material known as ceroid lipofuscin in Hermansky-Pudlak syndrome.

Methods Employed:

The principal methods employed are: 1) spectrophotometric, radiochemical, and fluorimetric enzyme assays; 2) cell culture techniques; 3) differential centrifugation; 4) quantitative ion exchange chromatography of amino acids in physiologic fluids; 5) radioisotopic measurements; 6) light, phase contrast, and electron microscopy; 7) column, thin layer, affinity chromatography of lipids and/or carbohydrates; 8) isoelectric focusing and polyacrylamide gel electrophoresis; 9) mass spectroscopy of unknown compounds; 10) pulsed amperometric (Dionex) detection of carbohydrates; 11) high performance liquid chromatography of dolichols and urinary oligosaccharides; 12) cloning and sequencing using plasmids, expression systems, and in vitro translation systems; 13) reverse-transcriptase PCR and direct PCR; 14) mutation analysis using Southern and Northern blotting and single-stranded conformational polymorphism (SSCP) analysis; 15) linkage analysis using polymorphisms in microsatellite repeats; 16) genomic DNA and expression library screening; 17) construction of yeast artificial chromosome contigs; 18) pulsed field gel electrophoresis; 19) use of radiation hybrid panels to determine the order of and distance separating markers on chromosome 17p.

Major Findings:

Cystinosis.
Nephropathic cystinosis is an autosomal recessive disorder due to defective transport of the amino acid cystine out of lysosomes. Cystinosis causes renal failure at approximately 10 years of age, but the cystine-depleting agent cysteamine retards or prevents this deterioration and enhances growth. Without cysteamine therapy, a variety of nonrenal complications plague post-transplant patients after their second decade of life.

Despite extensive knowledge concerning the medical and biochemical aspects of this disorder, neither the cystinosis gene nor the lysosomal cystine carrier has been isolated. Therefore, as part of a collaborative investigation with Dr. Mihael Polymeropoulos of the National Center for Human Genome Research and Dr. William van't Hoff of the Institute of Child Health, London, Dr. Geraldine McDowell has performed linkage analysis to determine that the cystinosis gene resides on the short arm of chromosome 17. In fact, the gene is linked to marker *D17S1584* with a maximal lod score of 10.89 at theta=0.03. These results are the culmination of a project involving 43 affected members of 23 cystinosis families from the United States, Northern Europe, Pakistan, and India and 328 different microsatellite markers. Homozygosity testing revealed that all affected offspring of the 4 cousin marriages included in the study were homozygous for *D17S1583* and *D17S1584*. Recombination events in two particularly informative families also placed the cystinosis gene in the 4 cM interval between *D17S1583* and *D17S1584*. There was no evidence of locus heterogeneity, and

both classical and adolescent cystinosis patients contributed positive lod scores, indicating that these two variants of cystinosis are allelic.

In collaboration with Drs. David Ledbetter and Akira Tanigami of the NCHGR, Drs. McDowell and Takao Isogai, a Special Volunteer, are further defining the boundaries of the cystinosis gene region, which contains no candidate genes. A YAC contig spanning the two flanking markers has been identified and both sequence-tagged sites (STS's) and polymorphic markers are being ordered on the YAC's by use of a radiation hybrid panel as well as CEPH families. Polymorphic repeats are being sought on the YAC's in order to use the recombinant cystinosis families to further narrow the cystinosis gene region, currently approximately 1 megabase in size. Concomitantly, the YAC's which comprise the cystinosis gene contig are being used to screen a chromosome 17-specific cosmid library as well as a cDNA library, to create a smaller contig which can be searched for expressing sequences. Candidate genes will be used as probes of Northern blots of a large number of patients' RNA. Investigators in the Section are also pursuing the transfection of YAC's or cosmids into transformed cystinosis fibroblasts to determine if correction of the basic defect (i.e., cystine storage) occurs. This would indicate the presence of the cystinosis gene on the transfected YAC or cosmid.

To maintain the Section's pre-eminence in the area of cystinosis, over 100 patients with this rare disease remain on the admission roster, to be seen at the NIH Clinical Center every two years for clinical followup. This year, several important developments have been reported by the group. First, children with cystinosis but without a renal transplant showed a very high incidence of nephrocalcinosis, probably due to their high concentrations of urinary phosphate and calcium, concomitants of the renal tubular Fanconi syndrome. Second, children well-treated chronically with cysteamine were shown to have a lower incidence of hypothyroidism than patients poorly treated or not treated at all. Third, the pharmacokinetics of cysteamine disappearance and cystine depletion after intravenous dosages of cysteamine were determined; cysteamine had a half-life of 20 min when administered at a dose of 10 mg/kg. Furthermore, the cystine depletion achieved by oral cysteamine equaled that for intravenous cysteamine, suggesting that oral cysteamine is completely absorbed by the gastrointestinal tract. Finally, Dr. Gahl continues to assist in the approval process for Cystagon in Europe, now that the Food and Drug Administration has approved this preparation for use in the United States.

In collaboration with Dr. Muriel Kaiser-Kupfer of the National Eye Institute, members of the Section continue to assist in the provision of cysteamine eyedrops, which dissolve the cystine crystals in patients' corneas. The NIH is the only source of these eyedrops for over 100 patients. Dr. Gahl also continues to investigate and treat the late complications of cystinosis (except those requiring a neurologist) in post-transplant patients.

Copper Metabolism.

The Section on Human Biochemical Genetics has investigated two inborn errors of copper metabolism, Menkes disease and Indian Childhood Cirrhosis.

Menkes disease is an X-linked disorder characterized by neurological death in the first three years of life due to impaired incorporation of copper into copper-dependent enzymes. The Menkes gene codes for a copper-transporting integral membrane protein with P-type ATPase activity. Dr. Stephen Kaler has previously employed reverse transcription PCR and other molecular biological techniques to delineate the mutations and residual mRNA expression of the Menkes disease gene in two families with mild Menkes disease or Occipital Horn Syndrome, a variant of classical Menkes disease. Recently, he has specifically correlated genotype with response to early copper histidine therapy in two different classical Menkes disease families. In one family, two related males began copper histidine treatment at 10 days of age and prenatally at 32 weeks gestation, respectively. A G to T transversion at the -1 exonic position of a splice donor site was identified, predicting a glutamine to histidine substitution at codon 724 of the Menkes copper-transporting ATPase gene. Like many other Menkes disease gene mutations, the Q724H mutation disrupted proper splicing; this mutation generated 5 mutant transcripts that skipped 1 to 4 exons. None of the resulting transcripts was

predicted to encode a functional copper transport protein. Copper histidine treatment normalized circulating copper and ceruloplasmin levels but did not improve the baseline deficiency of dopamine-β-hydroxylase, a copper-dependent enzyme. At 36 months of age, the first patient had neurodevelopmental abilities ranging from 10 to 15 months. The second patient also showed delayed neurodevelopment and died of pulmonary complications at 5 1/2 months of age. In this family, copper histidine therapy did not normalize neurological outcome, suggesting that the Q724H mutation did not preserve sufficient residual Menkes ATPase activity for significant clinical benefit to accrue from copper replacement.

In another Menkes disease family, Dr. Kaler detected a 5-base duplication at a splice acceptor site just upstream of a 226 bp exon in the middle of the Menkes coding sequence. One of the two transcripts resulting from this mutation contained a deletion of the preceeding 77 bp exon in addition to the 5 base insertion, creating an open reading frame. Consequently, RNAse protection studies revealed 25% of the normal Menkes disease transcript. This patient was treated with copper histidine from 8 days of age, and had normal head growth, brain myelination, and neurodevelopment including independent walking at 14 months of age. In contrast, his half-brother and first cousin with the same mutation were not treated with copper histidine from an early age and sufferred arrested head growth, delayed myelination, and severely delayed neurodevelopment. These results again emphasize that some residual functional copper transporting ATPase activity, in combination with early copper histidine therapy, is necessary for normal neurological development in Menkes disease.

Indian Childhood Cirrhosis (ICC) is a fatal hepatic disease characterized by storage of electron dense aggregates containing sulfur and copper. The basic defect is unknown, but Dr. Si Houn Hahn previously reported abnormal metallothionein synthesis in a single American boy with this disorder. Dr. Hahn subsequently demonstrated normal metallothionein synthesis in the cultured fibroblasts of several other ICC patients. This raised the possibility that some ICC patients may have mutations in another gene, specifically the Wilson disease gene, which codes for a copper-transporting ATPase presumed to function at the plasma membrane level. Dr. Ping Ye is now examining the 21 exons of the Wilson disease gene by single stranded conformational polymorphism analysis; she is sequencing several different exons which contain either polymorphisms or authentic mutations in the ICC cells. Making this distinction will require comparison with known Wilson disease gene mutations or expression studies.

Disorders of Carbohydrate Metabolism.

Dr. Donna Krasnewich continues to pursue the basic defects causing Carbohydrate Deficient Glycoprotein Syndrome (CDGS). This disorder is characterized by failure to thrive, psychomotor retardation, dysmorphic features, cerebellar atrophy, and serum N-linked glycoproteins which are improperly glycosylated. N-linked glycosylation involves the building of a dolichol-linked oligosaccharide tree, its transfer to the asparagine of a peptide chain, and subsequent processing from high-mannose to a "complex" form. Krasnewich, along with Dr. Gordon Holt, previously showed that two Danish CDGS type I patients had a metabolic defect early in the formation of dolichol-oligosaccharides. Specifically, their N-linked glycoproteins contained oligosaccharides with low ratios of N-acetylglucosamine to mannose and of galactose to mannose, a composition typical of early dolichol-oligosaccharides. Their cultured fibroblasts also exhibited decreased ^{3}H-mannose incorporation consistent with an inability to convent mannose into the intermediates which serve as substrates in the dolichol-oligosaccharide synthetic pathway. However, other CDGS patients with pathognomonic abnormalities in serum glycoprotein isoelectric focusing patterns did not demonstrate abnormal ^{3}H-mannose uptake in their fibroblasts. These patients also had N-linked glycoproteins whose oligosaccharides displayed normal N-acetylglucosamine to mannose and galactose to mannose ratios, indicating that a different metabolic defect is responsible for their disease. Dr. Krasnewich is currently documenting the biochemical heterogeneity in CDGS while investigating what specific step in N-linked glycosylation is impaired in each subgroup of affected individuals.

Drs. Geraldine McDowell and Gordon Holt are developing improved methods for analyzing the oligosaccharides excreted in the urine of patients with glycoproteinoses, glycolipidoses, and unknown

disorders of carbohydrate metabolism. The usual screening procedure for these disorders involves thin layer chromatography (TLC), which often gives ambiguous results. Since monosaccharide compositional analysis of carbohydrates provides a more powerful screening technique, McDowell and Holt developed a method of desalting urine on mixed-bed resins followed by trifluoracetic acid hydrolysis and analysis by HPLC. Results were normalized to creatinine concentration. The neutral oligosaccharides from 46 normal controls contained the following amounts of monosaccharides: fucose, 112 pmole/μg creatinine (range, 32-262); glucosamine, 405 (102-1203); mannose, 50 (0-295); galactose, 45 (0-327); and galactosamine, 165 (35-340). The amount of carbohydrate excreted by oligosaccharidosis patients was 3 to 70 times greater than that excreted by normal controls. Moreover, the monosaccharide patterns were unique for each individual disorder and allowed for the correct prediction of the enzyme deficiency. For example, patients with enzymatically proven α-mannosidosis excreted highly elevated amounts of oligosaccharides composed of glucosamine and mannose while the fucose, galactose, and galactosamine levels were normal. In contrast, patients with β-galactosidase deficiency excreted elevated amounts of glucosamine, mannose, and galactose with normal galactosamine levels.

Compositional analysis is also being employed to characterize abnormal oligosaccharides in disorders whose basic defect is unknown. Urine from two unrelated patients with geleophysic dysplasia contained moderate elevations of mucin-type oligosaccharides composed of fucose and N-acetylgalactosamine. Their fibroblasts showed evidence of lysosomal storage and will provide material for metabolic labeling studies. Urine from a patient with Schimke immuno-osseous dysplasia contained elevations of N-acetylglucosamine, a component of glycosaminoglycans, whose degradation can now be pursued. Another patient with an unknown lysosomal storage disorder has been found to excrete neutral oligosaccharides enriched in galactose. Several known enzyme deficiencies involving galactose have been ruled out, suggesting that this patient may represent a previously undescribed carbohydrate storage disease. Compositional analysis offers a significant improvement over TLC analysis of carbohydrates, and is being applied to a wide range of disorders of carbohydrate metabolism in the Section.

Finally, the Section remains the world's resource center for the diagnosis of new and established disorders of sialic acid metabolism. Samples from patients with a wide range of clinical presentations are analyzed by Dr. Krasnewich, and members of the Section have written the definitive chapter on sialidoses for the most authoritative reference text of neurologists, the Handbook of Clinical Neurology.

Melanosomal Tyrosine Transport.

The Section's longstanding interest in vesicular membrane transport has surfaced in a collaboration with Dr. Vincent Hearing and Brian Potterf, pigmentologists in the National Cancer Institute. Our two groups described a tyrosine and neutral amino acid transport system in the melanosomes of cultured melan-a murine melanocytes. The system closely resembles lysosomal system h of rat thyroid FRTL-5 cells, and is important because it explains how tyrosine, the initial precursor in the synthesis of the pigment melanin, is supplied to the interior of melanosomes for melanogenesis.

It has been proposed that the basic defect in a specific type of albinism, oculocutaneous albinism type II (OCA II), is failure of tyrosine to enter melanosomes. The OCA II gene has been cloned in humans; its murine counterpart is the *p* gene and the mutant phenotype is the pink-eyed dilution mouse. Our group investigated tyrosine transport in normal and pink-eyed dilution cultured murine melanocytes. Normal (melan-a) melanosome-rich granular fractions took up 10 μM ^{3}H-tyrosine at 21.1 ± 6.1 SEM pmol/min/mg protein (n=7) compared with 21.3 ± 5.8 SEM pmol/min/mg protein (n=5) for pink-eyed dilution, whose plasma membrane tyrosine transport was also normal ($K_m = 89\ \mu M$; $V_{max} = 302$ pmol/min/mg cell protein). We also found that pink-eyed dilution melanosomes were immature by virtue of their low density, high hexosaminidase activity, and lack of pigment. These data indicate that the *p* gene does not encode a tyrosine transporter of critical importance, and the basic defect in OCA II should be pursued elsewhere.

Rab Proteins.
Rab proteins are small GTP-binding proteins which play key roles in membrane trafficking, vesicle transport and organelle formation. Over 20 rabs have been isolated from rodents and 14 from humans. Some show tissue or cell specific expression and presumably are associated with cellular events characteristic of particular differentiated phenotypes. However, rab proteins associated with melanosomes or expressed in melanocytes have not been studied. Dr. David Chen in the Section designed two degenerate oligonucleotides corresponding to the invariant sequences of two GTP-binding domains in all known human rabs and used these oligonucleotides as primers to amplify RNA isolated from human melanoma cells in culture. Using this method, he isolated more than a dozen known rab and ras-related proteins, including rab 1, rab 3, rab 4, rab 8, rab 10, rab 11, rab 13, rap1A, rap 2A, and rho. These rabs, which accounted for the majority of clones, have been described before by others, but their expression in melanoma cells or melanocytes had not been examined.

Dr. Chen also identified four novel cDNA clones. One was the human homologue of a rat small GTP-binding protein gene, ram, which was initially cloned from a rat megakaryocyte cDNA library. Another cDNA had a sequence that perfectly matched that of a human small GTP-binding protein, c25KG, which was initially purified from platelets but was only partially sequenced. These two cDNAs showed characteristic features of rab proteins and shared a high degree of homology with each other (80%), but had minimal homology with other rabs (less than 30%). These results indicated that the two cDNAs are in a distinct rab subfamily and, although first identified from megakaryocytes and platelets, they are also expressed in melanocytes. The other two cDNAs also contained the signature residues of rab proteins, but only showed moderate homology with any of the known mammalian rabs, suggesting that they represent new members of the rab family.

Two autosomal recessive diseases, Hermansky-Pudlak syndrome (HPS) and Chediak-Higashi disease (CHD), exhibit morphological changes of cellular vesicles, i.e., lysosomes, melanosomes, and platelets or platelet dense bodies. These two disorders may result from abnormal intracellular membrane traficking or vesicular targetting, processes which may be mediated by rab proteins. Therefore, in collaboration with Dr. Ray Boissy of the University of Cincinnati College of Medicine, Dr. Chen is examining poly A+ RNA from cultured melanocytes of normal individuals as well as patients with HPS and CHD. He will perform RT-PCR using as primers pairs of specific oligonucleotides for each human rab known to be expressed in melanocytes and then for the novel rab-related sequences isolated in our lab. This will allow him to examine whether a rab gene is expressed in the normal and mutant melanocytes and to generate probes for northern blot analysis which will help quantitate the levels of each rab isoform expressed in the various types of melanocytes.

Hermansky-Pudlak Syndrome.
Hermansky-Pudlak syndrome (HPS) consists of the triad of albinism, a bleeding diathesis, and lysosomal storage of ceroid lipofuscin. The albinism is tyrosinase-positive, and of extremely variable severity. The bleeding diathesis is due to a platelet storage pool defect. Specifically, the platelet dense bodies, which contain ADP, ATP, calcium, and serotonin, are virtually absent in HPS. These intracellular vesicles are responsible for the secondary aggregation response of platelets. Ceroid lipofuscin is a poorly-defined, lipid-protein substance stored inside lysosomes which fluoresces in a characteristic fashion. It appears to accumulate progressively in HPS. The basic defect remains unknown, but probably involves the membranes of the melanosome, dense body, and lysosome, all of which are impaired in HPS. This autosomal recessive disease is most common in Puerto Rico, where the incidence in some areas is 1 in 1800 live births. Clinical manifestations are extremely variable, but the most serious and insidious medical complication is usually pulmonary fibrosis, presumably due to the inexorable accumulation of ceroid lipofuscin. Another organ affected by the storage is the intestine, which manifests a granulomatous colitis in some patients. Diagnosis of HPS rests upon finding absent or rare platelet dense bodies in an albino patient with a storage pool defect.

In order to determine the basic defect in HPS, members of the Section sought to identify the stored substance in the cellular lysosomes of a 7-year old girl with HPS and granulomatous colitis. We first verified that the ceroid lipofuscin was present in sloughed renal tubular cells by showing that they

fluoresced in a typical fashion, while normal renal tubular cells did not. Then Dr. Juanru Guo performed lipid extraction of the renal cells followed by thin-layer chromatography, and identified a spot present in the patient's sloughed cells but not in normal cells. This spot migrated with authentic dolichol, a lipid composed of 15-23 isoprene units, i.e., 75-115 carbons. The compound was identified by mass spectroscopy (Dr. Henry Fales, NHLBI) as C_{85} dolichol, and HPLC of several HPS urines confirmed a quantitative increase in dolichols in the sloughed renal tubular cells of 7 of 8 individuals with the disorder. Our index case also exhibited an elevated plasma concentration of carotene, which is composed of isoprene units, and of cholesterol itself. Since dolichol, carotene, and cholesterol all derive from the cholesterol synthetic pathway, members of the Section are studying the enzymes of this pathway for possible constitutive up-regulation. The *in vitro* model systems employed include fibroblasts, in collaboration with Dr. Skip Waechter of the University of Kentucky, and melanosomes, in collaboration with Dr. Ray Boissy of the University of Cincinnati.

Significance to Biomedical Research and the Program of the Institute:

This Section's investigations, directed toward the etiology, diagnosis, and treatment of inborn errors of metabolism, fulfill important objectives of biomedical research in general and address primary concerns of the Institute. The Section serves as a widely recognized repository for clinical and biochemical expertise in the field of cystinosis, for biochemical analysis of disorders of sialic acid metabolism, and for methodology in the fields of carbohydrate analysis and lysosomal membrane transport. Regarding cystinosis, the Section follows over one-third of all the known patients in the United States and serves as the sole source of cysteamine eyedrops (through the National Eye Institute) for over 100 patients. The Section's studies on the cloning of the lysosomal membrane's cystine transporter will permit the different mutations in cystinosis to be defined at the molecular level, and will help determine how carriers targeted for the lysosomal membrane reach their final destination.

The Section's pursuit of copper histidine therapy for young Menkes disease patients offers the hope that some of the symptoms of this devastating disorder can be ameliorated. Determination of the particular mutations involved in different families now allows for heterozygote diagnosis in specific families. The development of new analytical methods for disorders of carbohydrate metabolism will improve the diagnosis of known and unknown lysosomal storage diseases. The Section's investigations into other disorders, including Carbohydrate Deficient Glycoprotein Syndrome and Hermansky-Pudlak Syndrome, should reveal new pathways in human cell biology, and may allow for new methods of diagnosis and treatment for these rare human conditions.

Proposed Course:

Future efforts will include: 1) Attempts to isolate the cystinosis gene by using YAC and cosmid contigs, positional cloning, and selection of originally cystinotic cells cured by cDNA transfection; 2) Mutational analysis of cystinosis patients once the gene is cloned; 3) Continuation of clinical followup of cystinosis patients every 2 years; 4) SSCP and sequencing of Wilson disease gene exons in Indian Childhood Cirrhosis fibroblasts; 5) Analysis of the content and structure of the carbohydrates of N-linked glycoproteins in Carbohydrate-Deficient Glycoprotein Syndrome, with specific attempts to identify the mutant enzyme involved in dolichol-oligosaccharide synthesis in type I patients; 6) Use of newly-developed methodologies in urinary carbohydrate analysis to define the basic defects in a variety of carbohydrate disorders of unknown etiology; 7) Determination of the pattern of expression of rab genes in human melanocytes cultured from normal, HPS, and CHD patients; 8) Investigation into the basic defect in Hermansky-Pudlak syndrome, specifically the cause of ceroid lipofuscin storage, using fibroblasts and melanocytes of affected patients to study the cholesterol synthetic pathway.

Clinical Trials:

Protocols of the Section on Human Biochemical Genetics currently approved by the NICHD Institutional Review Board include:

78-CH-93 - "Use of Cysteamine in the Treatment of Cystinosis"

76-CH-238 - "Diagnosis and Treatment of Patients with Inborn Errors of Metabolism"

94-CH-201- "Glutathione Monoethyl Ester in the Therapy of Human Glutathione Synthase Deficiency"

92-CH-177 - "Studies of the Genetic Defect in Indian Childhood Cirrhosis on the Indian Subcontinent"
Terminated; no patients in past year.

90-CH-149 - "Copper Histidinate Therapy in Menkes' Disease"

88-CH-26 - "Clinical and Biochemical Evaluation of Oculocerebrorenal Syndrome of Lowe"
Terminated; no patients in past year.

William A. Gahl, MD, PhD, is the principal investigator for the first four protocols. The glutathione ethyl ester protocol is directed by William Rehead of the University of Iowa and involves two patients studied at the NIH Clinical Center. In addition, Dr. Gahl is associate investigator for the other protocols and for National Eye Institute project 86-EI-62, "A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine in the Treatment of Corneal Cystine Crystal Accumulation in Cystinosis". Stephen G. Kaler, MD, is principal investigator of the otocol 90-CH-149.

Principal Investigator: William A. Gahl, M.D., Ph.D.
Heritable Disorders Branch, NICHD

Title:

76-CH-238 - "Diagnosis and Treatment of Patients with Inborn Errors of Metabolism"

Hypothesis: This is a screening and training protocol to determine if patients meet entry criteria for other protocols, to provide fellows with experience in cases of rare metabolic disorders, and to examine patients with unknown diseases to decide if pursuit of their basic defect is warranted.

Study Design: Diagnosed and undiagnosed patients are thoroughly examined and followed periodically as inpatients or outpatients, using currently accepted clinical testing procedures.

Current Results: One patient with Hermansky-Pudlak syndrome seen under this protocol instigated the initiation of an in-depth clinical project to study the cause of this disease. In contrast, a boy with cerebellar ataxia yielded no clue to the etiology of his disorder, and he will be followed annually without further pursuit of his disease entity.

Conclusions: The protocol provides an appropriate avenue to examine selected patients pertinent to the studies of the Branch.

Principal Investigator: William A. Gahl, M.D., Ph.D.
Heritable Disorders Branch, NICHD

Title:

78-CH-93 - "Use of Cysteamine in the Treatment of Cystinosis"

Hypothesis: Nephropathic cystinosis is an autosomal recessive disorder due to defective transport of the amino acid cystine out of lysosomes. Cystinosis causes renal failure at approximately 10 years of age, but the cystine-depleting agent cysteamine retards or prevents this deterioration and enhances growth. Without cysteamine therapy, a variety of nonrenal complications plague post-transplant patients after their second decade of life. Since 1978, the hypothesis that cysteamine could prevent or attenuate the various clinical manifestations of cystinosis has been tested in scores of patients admitted under this protocol.

Study Design: Approximately 50 pre-transplant patients with nephropathic cystinosis receiving oral cysteamine (currently, Cystagon) are admitted to the NIH Clinical Center every 2 years for followup of their condition. Measurements of renal function, growth, and development are monitored for comparison with the natural history of the disease, which has been recorded previously in patients not receiving cysteamine. Approximately 50 post-transplant patients are examined every 2 years to record the occurrence and severity of late complications of cystine storage.

Current Results: Children with cystinosis but without a renal transplant showed a very high incidence of nephrocalcinosis, probably due to their high concentrations of urinary phosphate and calcium, concomitants of the renal tubular Fanconi syndrome. Children well-treated chronically with cysteamine were shown to have a lower incidence of hypothyroidism than patients poorly treated or not treated at all. The pharmacokinetics of cysteamine disappearance and cystine depletion after intravenous dosages of cysteamine were determined; cysteamine had a half-life of 20 min when administered at a dose of 10 mg/kg. Furthermore, the cystine depletion achieved by oral cysteamine equaled that for intravenous cysteamine, suggesting that oral cysteamine is completely absorbed by the gastrointestinal tract. In studies of post-transplant patients, it was determined that affected individuals over 30 years of age all had some debilitating complication of the disease such as retinal blindness, a vacuolar myopathy, or cortical atrophy and calcifications.

Conclusions: Cystinosis is a multisystemic disease. The cystine-depleting agent cysteamine, which retards or prevents renal glomerular deterioration, enhances growth, and prevents hypothyroidism, has now been approved for use in the United States by the Food and Drug Administration. It represents the treatment of choice for nephropathic cystinosis, and presumably will prevent the late complications of the disease in patients treated from an early age.

Prinicpal Investigator: Stephen G. Kaler, M.D.
Heritable Disorders Branch, NICHD

Title:

90-CH-149 - "Copper Histidinate Therapy in Menkes' Disease"

Hypothesis: Menkes disease is an X-linked disorder characterized by neurological death in the first three years of life due to impaired incorporation of copper into copper-dependent enzymes. The Menkes gene codes for a copper-transporting integral membrane protein with P-type ATPase activity. This study tests whether early therapy with copper histidine can allow for normal neurodevelopment in Menkes disease patients.

Study Design: Children with Menkes disease are treated with copper histidine, 250 μg q day subcutaneously, as soon after birth as diagnosis permits. Outcome parameters include serum copper and ceruloplasmin, head circumference, growth, and neurodevelopment.

Current Results: One patient with 25% residual function of his copper-transporting ATPase, as assessed by expression of the Menkes disease gene, responded to copper replacement therapy by displaying normal development at 14 months of age. Other patients, with no evidence for normal Menkes gene transcripts, did not benefit significantly from copper replacement therapy.

Conclusions: These results emphasize that some residual functional copper transporting ATPase activity, in combination with early copper histidine therapy, is necessary for normal neurological development in Menkes disease. (This protocol has been transferred to NINDS.)

Principal Investigator: William A. Gahl, M.D., Ph.D.
Heriatble Disorders Branch, NICHD

Title:

94-CH-201- "Glutathione Monoethyl Ester in the Therapy of Human Glutathione Synthase Deficiency"

Hypothesis: This study, which recapitulates a protocol approved and funded for Dr. William J. Rhead of the University of Iowa, investigates the hypothesis that glutathione (GSH) monoethylester will be efficacious in the treatment of the inherited metabolic disorder, glutathione synthetase deficiency (GSD). Affected patients have chronic metabolic acidosis, chronic hemolytic anemia, and late onset, progressive mental deficiency. The aim is to restore intracellular GSH levels to 40-60% of normal, a level expected to reverse the clinical and biochemical sequelae of this disorder.

Study Design: GSH monoethylester is administered for two 4-month periods, separated by a 4-month wash-out period, to two NIH patients (brothers) who have GSD. The starting dose is 1 mmol/kg.day divided into four oral doses, to be advanced by 1 to 2 mmol/kg/day every week to a maximum of 10 mmol/kg/day. Acid-base balance, serum electrolytes, plasma amino acids, urinary oxoproline excretion, RBC ergothioneine levels, hematologic parameters, hepatic, renal, ophthalmologic and developmental functions are monitored throughout the study.

Current Results: Only one brother agreed to the study. He tolerated at most 1.5 mmol/kg/day of GSH monoethylester orally, a dose above which he vomited. At this dosage, there was no increase in red blood cell or white blood cell glutathione, and daily 5-oxoproline excretion did not decrease significantly. There were no abnormal laboratory values attributable to the treatment, and clinical side efects were limited to acute nausea and vomiting after ingestion of the drug.

Conclusions: Side effects may limit the clinical efficacy of this drug, but Dr. Rhead has further pursued increased dosing by nasogastric tube to this patient and another GSD patient at the University of Iowa.

Publications:

Andersson HC, Kohn AD, Gahl WA, Kohn LD. Photoaffinity labeling of lysosomal membrane proteins with $[^{125}I]$-diiodotyrosine, a system h ligand. Biochem Molec Med 1995 (In press).

Gahl WA. Lysosomal Storage Diseases. In: Burg FD, Ingelfinger JR, Wald ER, Polin RA, eds. Current Pediatric Therapy. 15th ed. New York: WB Saunders Co. (In press).

Gahl WA, Ingelfinger J, Mohan P, Bernardini I, Hyman PE, Tangerman A. Intravenous cysteamine therapy for nephropathic cystinosis. Pediatr Res 1995 (In press).

Gahl WA, Krasnewich DM, Williams JC. Sialidoses. In: Vinken PJ, Bruyn GA, eds. Handbook of Clinical Neurology. Amsterdam: Elsevier, 1995, chapter 25 (In press).

Gahl WA, Potterf B, Durham-Pierre D, Brilliant MH, Hearing VJ. Melanosomal tyrosine transport in normal and pink-eyed dilution murine melanocytes. Pigment Cell Res 1995 (In press).

Gahl WA, Schneider JA, Aula P. Lysosomal transport disorders - Cystinosis and sialic acid storage diseases. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic Basis of Inherited Disease. 7th ed. New York: McGraw-Hill, 1994 , 3763-97.

Hahn SH, Krasnewich DK, Brantly M, Kvittingen EA, Gahl WA. Heterozygosity for an exon 12 splicing mutation and W234G missense mutation in an American child with chronic tyrosinemia type I. Hum Mutat 1995; 6:66-73.

Hahn SH, Tanner MS, Danks DM, Gahl WA. Normal metallothionein synthesis in fibroblasts obtained from children with Indian Childhood Cirrhosis or copper-associated childhood cirrhosis. Biochem Molec Med 1995; 54:142-5.

Kaiser-Kupfer MI, Chan C-C, Markello TC, Crawford MA, Caruso RC, Csaky KG, Guo J, Gahl WA. Clinical biochemical and pathologic correlations in Bietti's crystalline dystrophy. Am J Ophthalmol 1994; 118:569-82.

Kaler SG. Copper histidine therapy in Menkes disease: clinical, biochemical and molecular aspects. In: Sarkar B, ed. Genetic Response to Metals. New York: Marcel Dekker, Inc. (In press).

Kaler SG, Buist NRM, Holmes CS, Goldstein DS, Miller RC, Gahl WA. Early copper therapy in classical Menkes disease patients with a novel splicing mutation. Annals Neurol 1995 (In press).

Kaler SG, Gallo LK, Proud VK, Percy AK, Mark Y, Segal NA, Goldstein DS, Holmes CS, Gahl WA. Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. Nature Genetics 1994; 8:195-202.

Kimonis VE, Troendle J, Yang ML, Rose SR, Markello TC, Gahl WA. Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. J Clin Endocrin Metab 1995 (In press).

Krasnewich DK, Holt GD, Brantly M, Skovby F, Redwine J, Gahl WA. Abnormal synthesis of dolichol-linked oligosaccharides in Carbohydrate-deficient Glycoprotein Syndrome. Glycobiology 1995 (In press).

McDowell GA, Gahl WA, Stephenson L, Schneider J, Weissenbach J, Polymeropoulos MH, Town MM, van't Hoff W, Farrall M, Mathew CG. Linkage of the gene for cystinosis to markers on the short arm of chromosome 17. Nature Genetics 1995; 10:246-8.

Schneider JA, Clark KF, Greene AA, Reisch JS, Markello TC, Gahl WG, Thoene JG, Noonan PK, Berry KA. Recent advances in the treatment of cystinosis. J Inher Metab Dis 1995 (In press).

Theodoropoulos DS, Shawker TH, Heinrichs C, Gahl WA. Medullary nephrocalcinosis in nephropathic cystinosis. Pediatr Nephrol 1995 (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00137-21 HDB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization, Regulation, and Expression of the UDP-Glucuronosyltransferase Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ida S. Owens	Head	HDB, NICHD
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	Nahid Gholamai	Visiting Fellow	HDB, NICHD
	Christine Potter	Technician	HDB, NICHD

COOPERATING UNITS *(if any)*

Imperial College of Science, Technology, and Medicine, London, England (Dr. D.R. O'Reilly)
University of Rochester, Department of Pharmacology, (Dr. J. P. Jones).

LAB/BRANCH

Heritable Disorders Branch

SECTION

Genetic Disorders of Drug Metabolism

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.3	3.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our description of the *UGT*1 locus has proven critical for the determination of genetic defects in patients with Crigler-Najjar(CN) diseases. The original version of the gene complex (*UGT1A-UGT1F*) codes for 2 bilirubin (Br), 3 Br-like, and 1 phenol transferases. In the 5' region, six different exons 1, each with an upstream promoter and each encoding the amino terminus of an isoform, are arrayed in series with 4 common exons encoding six identical carboxyl termini in the 3' region of the locus. By way of extending the locus seven cosmid clones with at least six additional exons 1 are being mapped for restriction endonucleases and analyzed by Southern blot hybridization. The six exons have been sequenced and tentatively arranged in the series. The newly identified exons are related to the unique region of a second class of phenol isoforms typified by HLUG P4 that preferentially metabolizes bulky phenols. Out of 13 different deleterious mutations in the *UGT1A* gene of CN patients, 4 missense ones in CN-I and 4 in CN-II patients proved to either completely inactivate or partially inactivate HUG-Br1, respectively, accounting for the CN-II phenotype. A deleterious phenylalanine codon-180 deletion mutant (CN-I) illuminated a conserved hydrophobic Micro-region A (MR A, codons 161-180) which, according to computer analysis, is a buried hydrophobic helical structure predicted to be associated with membrane and is unique to the bilirubin isoform. A missense mutation, G276, in a CN-I patient disrupted a strictly conserved di-glycine (codons 276/277) in a conserved Micro-region B (MR B, codons 270-288) in a region predicted to form a β-hair pin loop; the mutation completely abolished activity. The conservation of critical residues and MR-B between HUG-Br1 and the soluble but related baculoviral UDP-glucosyltransferase (egt) support the claim that the viral protein could serve as a structural model for the mammalian bilirubin isoform. Further, two different missense mutations in one allele of the acetaminophen-selecting *UGT1F*-encoded HLUG P1 were uncovered in the heterozygous state of genomic DNA of an apparent unaffected individual, promising to be the first defect uncovered at a transferase locus that affects drug metabolism.

Project Description

Objectives:

The primary objectives of this project are:

To carry out chromosomal walking upstream of the original version of the *UGT1* locus to select overlapping cosmid clones in order to locate other exons 1 encoding additional mRNAs with the 3' common exons at this locus.

To carry out restriction enzyme and sequence analyses of cosmid clones which overlap and extend the *UGT1* locus.

To determine the defects among as many Crigler-Najjar, Types I and II, and Gilbert's individuals, as possible.

To use the single-stranded conformational polymorphisms (SSCP) for rapid screening of Crigler-Najjar individuals and Gilbert's individuals to locate defects at the *UGT1* locus.

To use PCR-amplification and PCR-sequencing to define specific alterations uncovered by the SSCP technique.

To make missense mutations in the normal bilirubin or phenol transferase cDNAs (isolated in our laboratory) consistent with each missense mutation uncovered in the genome of patients with defective bilirubin/phenol glucuronidation.

To express individually mutated bilirubin transferase cDNAs in COS-1 cells either transiently by using recombinant plasmids or a stably expressing Hep.G2 cell line using recombinant vaccinia viral constructs.

To determine the structure-function relationship of each mutation of bilirubin transferase activity by using a ^{14}C-labeled substrate in an *in vitro* assay which separates each of the three bilirubin glucuronides or by increasing the sensitivity by developing and analyzing glucuronides on a HPLC system.

To use biochemical techniques to analyze a unique hydrophobic region and a protein β-loop in the major bilirubin isoform for effects on glucuronidating activity.

To isolate and characterize cDNAs for all viable new exons 1 uncovered at the *UGT1* locus, testing a range of therapeutic and nontherapeutic chemicals.

To purify the baculoviral ecdysone UDP-glucosyltransferase (egt) using a recombinant egt cDNA-viral construct supplied by a collaborator (Dr. D. R. O'Reilly) for expression in either the SF9 insect cell, a bacterial GST gene fusion system, or the pPIC9 gene fusion system for expression in the *Pichia pastoris* yeast system.

To use the egt protein in crystallographic studies (carried out in collaboration with Dr. J. Jones) to determine its x-ray diffraction pattern for tertiary structural analysis.

To carry out computer modeling of the crystallographic structure of the egt protein and to derive a conformation for the analogous bilirubin UDP-glucuronosyltransferase using the results from our structure-function relationship studies.

To use the flanking regions from the major and minor bilirubin transferase genes contained in the *UGT1* complex in connection with a reporter-gene to locate regulatory elements.

To carry out deletions and/or site-directed mutagenesis to establish precise regulatory nucleotide sequences to be used in connection with the detection of nuclear regulatory factors.

To isolate and use mRNA from liver and kidney of variously treated monkeys (provided to us by a

collaborator) to establish whether any of the species encoded at the *UGT1* complex is inducible and, if so, by which effector compounds.

To construct vectors containing retroviral promoter elements/enhancers containing the HUG-Br1 or the HUG-Br2 cDNA for the stable expression of bilirubin transferase activity in Hep.G2 cell.

To make specific synthetic peptides of the two bilirubin transferase proteins for the development of monoclonal antibodies.

To determine mRNA tissue distribution for the newly uncovered *UGT1*-encoded transferase isoforms to explore the physiological consequences of isoform distribution and, thus, function.

Methods Employed:

The principal methods employed are: 1) Enzyme assays using spectrophotometric, spectrophotofluorometric, and radiometric determinations of products separated by TLC chromatography; 2) HPLC Chromatography; 3) Cloning of cDNAs into phage and plasmid vectors; 4) Sublconing of cDNA and genomic DNA fragments; 5) Polymerase chain reactions to synthesize ample quantities of specific DNA; 6) Single-strand and double-strand DNA sequencing using the PCR technique; 7) Computer analysis of nucleotide sequence data; 8) Agarose gel electrophoresis of DNA and RNA; 9) Filter hybridization of DNA probes using Southern, Northern, slot, and dot blot techniques; 10) Site-directed mutagenesis of critical bases to test the effect of missense and selected alterations on activity; 11) Restriction-enzyme mapping of genomic DNA; 12) Isolation of DNA inserts in plasmid and phage by endonuclease treatment, extraction, and centrifugation; 13) Transformation of yeast and transfection of COS-1 and Hep.G2 cells with expression vectors containing human transferase cDNA inserts; 14) Tissue culture techniques; 15) Western blot analysis of transferases; 16) Monoclonal antibody production using synthetic peptides based on deduced amino acid sequences derived from cDNA sequence data; 17) Immunoprecipitation of transferase-specific proteins directed at specific determinants including possible acceptor substrate, as well as donor substrate, binding sites; 18) construction of target DNA-reporter gene fusion systems for analysis of nucleotide regulatory elements in genes; 19) transfection studies of target DNA-reporter gene fusion constructs into appropriate cell lines; 20) construction of deletion mutants to locate regulatory nucleotide sequences; 21) Purification of enzyme protein by cation exchange chromatography and gel filtration; (22) computer modeling of the tertiary structure of the related baculoviral UDP-glucosyltransferase to derive a conformation of the human bilirubin isozyme.

Major Findings:

The *UGT1* gene complex encoding both bilirubin and phenol UDP-glucuronosyltransferases (transferase) has been isolated and partially characterized. Three overlapping cosmid clones selected with the unique 5'regions of the bilirubin transferase cDNAs contained the original version of the complex locus (*UGT1A* - *UGT1F*) which codes for 2 bilirubin, 3 bilirubin-like, and 1 phenol transferases spanning some 95 kb. Six different exons 1, each with an upstream promoter element and each encoding the unique amino terminus of an isoform, are arrayed in series with 4 common exons in the 3' region of the locus that encode six identical carboxyl termini of the transferase isoforms. Exons 1A and 1D encode the unique region of the major and minor bilirubin transferase cDNAs, HUG-Br1 and HUG-Br2, respectively; exon 1F encodes the same region of the cDNA of a phenol isoform, HLUG P1, reported earlier in the literature. Exons 1BP, 1C, and 1E were uncovered at the gene level for the first time and encode bilirubin-like (HUG-Br2-like) isoforms. Exons 2-5 encode the common carboxyl terminus of every isoform. The most 5'exon was used to select other cosmid clones to extend the locus by more than 310 kb. The total size of the locus is more than 410 kb: 95 kb for the original version and a minimal of 310 kb for the extended portion. We have identified six additional exons 1 (bringing the total to 12) contained in 7 cosmid clones; the clones are undergoing restriction endonuclease-mapping and Southern blot hybridization analysis. The six exons have been sequenced and, tentatively, arranged in a series. Due to a high degree of identity for the last six exons and, thus, all exonic probes, jumping along the locus has contributed to gaps. We continue to identify unique intronic sequences for probes to create overlap of the cosmid inserts.

The newly identified exons form a group highly related to the unique region of a second class of phenol isoforms typified by the HLUG P4 isoform reported in the literature and shown to prefer bulky phenolic substrates. Since bilirubin is an acyl-glucuronide, it is expected that some of the newly uncovered exons

at this locus will encode transferases involved in acyl glucuronidation and idiosyncratic metabolism of certain therapeutic drugs, including the nonsteroidal anti-inflammatory drugs (NSAIDS). In certain subpopulations of individuals unidentified UDP-glucuronosyltransferase(s) form the highly reactive acyl glucuronides of certain NSAIDS and other chemicals which undergo rearrangement to generate "leaving groups" that attack and covalently bind to proteins forming haptenes and, subsequently, the development of severe autoimmune responses. The acyl bilirubin-glucuronide rearranges but does not form reactive leaving groups. The extent of overlapping substrate specificity of the bilirubin isoform(s) for these types of drugs or the substrate specificity of these new isoforms remains to be determined.

In addition to uncovering new exons 1, we have shown that *UGT1C* among the *UGT1A-UGT1F* complex is expressed constitutively upon analyzing some 11 different human mRNA samples. The bilirubin-like isoforms have been constructed and the clones are now ready for expression to analyze for substrate specificity of the isozymes. Similarly, the expression of mRNA for the second group of exons 1 is under investigation.

The characterization of the *UGT1* locus, encoding multiple bilirubin transferase isozymes, allowed us to uncover 13 different mutations in the *UGT1A* gene encoding the major bilirubin transferase isozyme. Of the eight missense mutations uncovered, we have shown that the 4 seen in CN-I patients completely inactivated the major bilirubin transferase activity at pH 6.4 and that the 4 seen in CN-II patients partially inactivated the enzyme accounting for residual activity in CN-II patients.

A new class of mutations involving the TATA box transcriptional element was found in both CN-I and CN-II individuals. In two *UGT1A*-heterozygous individuals either a TA was deleted in a CN-II individual or a TA was inserted in a CN-I patient at the unusual wild-type TATA box element consisting of TA_7A. We are currently preparing mutants to assess the effect of the mutations on transcription. Together the results suggest a spectrum of clinical phenotypes among patients. The inheritance of all the bilirubin-related diseases, previously considered unclear, could be accounted for by recessive inheritance of combinations of defects culminating in the phenotypes observed in the population of CN-I, CN-II and Gilbert's individuals.

A deleted phenylalanine codon at position 170 in exon 1 of *UGT1A* has enabled us to identify a hydrophobic region designated microregion A. The mutation disrupted a conserved di-phenylalanine among the bilirubin/bilirubin-like (*UGT1A-UGT1E*) isoforms. Studies with mutant HUG-Br1 protein derived from the pΔF170HUG-Br1 cDNA expression unit transfected into COS-1 cells demonstrated pH sensitivity with respect to generating product; at the routinely used pH (7.6) for bilirubin glucuronidation both the mutant and wild type proteins had similar activities. HUG-Br1 showed 3-4 fold more activity at pH 6.4 than at pH 7.6, and the mutant had no activity at pH 6.4. The pH dependency of human liver microsomal bilirubin transferase activity was similar to that of HUG-Br1.

Using the RAO-ARGOS computer program designed to detect buried helix in membrane-anchored proteins, we discovered that the membrane-bound HUG-Br1 protein contains a buried helix in microregion A, a structure shown to be unique to bilirubin isoforms. Peak hydrophobicity centers around the 170/171 di-phenylalanine. Prompted by the unique hydrophobic characteristics of this region in the HUG-Br1 type proteins, we made select amino acid changes in this region and analyzed for structure-function relationship of the substitutions. Depending upon the amino acid in the 170/171 di-phenylalanine and of other hydrophobic residues in the region, the RAO-ARGOS program reflected effects on the buried helix. Mutant proteins with Phe-170 conversion to Leu, Iso, Val, or Ala and either Pro-167 or Pro-176 to Gly caused a total loss of activity. Leu-171, but not Ile-171, was active although both satisfied the requirements to form a buried helix, indicating that the amino acids in the predicted helical structure are important for activity. His-173 converted to Arg, had activity comparable to the wild-type. If one considers the three major groups of transferase isoforms, the buried helix can be accounted for by differences in hydrophobic amino acids in this region of the transferase isoforms. Analysis of the bilirubin-type and the phenol isoforms in this region demonstrated that the bilirubin-type has 2 consecutive aromatic residues out of 4 consecutive hydrophobic residues, compared to the phenol forms with 2 alternate aromatic residues or one aromatic out of 4 hydrophobic residues. A comparison of this same region in steroid isoforms shows that a Ser or Thr, which preceded the 4 consecutive hydrophobic residues in the bilirubin/phenol isoforms, appeared to have translocated downstream to split the 4 consecutive hydrophobic residues to 3 and 1 and contained no consecutive aromatic ones. The rank in hydrophobicity of this microregion A between these three substrate types is: bilirubin > phenol > steroid

which is consistent with the high hydrophobicity to generate a buried helix in the bilirubin isoform as determined by the RAO-ARGOS program. That is --this hydrophobic region that is critical for bilirubin transferase activity-- appears to reflect an appropriate hydrophobic milieu for binding/interacting with the extremely hydrophobic-behaving bilirubin substrate.

The characterization of this unique buried helix and its apparent membrane-association in the bilirubin isoform may explain the poor restitution of bilirubin transferase activity during purification strategies and the problematic assay for bilirubin turnover.

Since 17α-ethynlestradiol (EE) was shown in the literature to be a good substrate for the HUG-Br1 isoform, we analyzed the pH dependency of EE catalysis by this protein. The HUG-Br1 generated 1.5 fold more EE-glucuronide at pH 6.4 than at pH 7.6. The pH 6.4 requirement is quite sharp for bilirubin, whereas EE has a broad pH range in this region. The Km for EE is 25 μM compared to 5 μM for bilirubin, but the relative turnover number for EE/bilirubin is 5 to 10. The protein distinguished the substrates with respect to heat stability; HUG-Br1 subjected to 50°C between 1 to 5 min showed that bilirubin turnover is completely loss after 1 min whereas 50 % of its activity for EE remains after 5 min. Each of the mutant proteins described above, except P176G, failed to turnover EE, a pattern similar to that for bilirubin. Although HUG-Br1P176G failed to glucuronidate bilirubin, the turnover of EE was 50% of that for wild-type. The studies indicate that EE is a superior substrate for HUG-Br1 and appears to undergo catalysis at a different site or, at best, overlapping active sites for the two substrates. In spite of the apparent lower turnover and increased heat lability for bilirubin turnover, the HUG-Br1 gene and enzymology account for the clinical phenotypes in defective bilirubin detoxification.

In a second CN-I patient we uncovered a point mutation at codon 276 (G-->C), in the unique exon 1 of *UGT1A* which converted a Gly to an Arg (G276R) disrupting a conserved di-glycine at position 276/277 in the HUG-Br1 protein and bilirubin-like isoforms. The mutant protein had no detectable bilirubin glucuronidating activity at either the routinely used pH 7.6 or at the recently uncovered major pH optimum of 6.4. Although each of the bilirubin-type isozymes contains a conserved peptide structure between residues 270-288 composed of 4 α-helix-breaking-, 5 amide-containing-, and 7 large hydrophobic residues, all UDP-glucuronosyltransferases contain the di-glycine peptide within this region at approximately position 276-277, making it strictly conserved despite its coding by a unique exon 1 at the *UGT1* locus. We designate residues between 270-288 as microregion B. The structure-function relationship in this region was studied by site-directed missense mutations in the HUG-Br1 cDNA as follows: G276A, G276Q, G276E, G276I, and P270G as well as V275I and P285G. The first set of mutations completely abolished glucuronidating activity, and the latter set had no effect on activity at either pH value. Conservation of residues between the related baculoviral ecdysone UDP-glucosyltransferase (egt) and the UDP-glucuronosyltransferases confirms the important role of the Gly-276 as well as other residues. The strict conservation of the di-peptide in all UDP-glucuronosyltransferase, its retention in the distant and related baculoviral ecdysone UDP-glucosyltransferase, and the context of the residues in the proteins suggest that the di-glycine peptide is part of a critical β-hair pin loop structure, no doubt, required in all UDP-glucuronosyltransferases as well as the UDP-glucosyltransferase.

Since the soluble baculoviral ecdysone UDP-glucosyltransferase protein contains the apparent β-loop structure in the unique amino terminus, as well as 61% / 21% sequence similarity/identity within the equivalent common-end segment (excluding the final 30 amino-acid for membrane-anchoring), we are using the UDP-glucosyltransferase as a model for crystallization studies as a strategy to gain information on the active site of the human bilirubin transferase.

Due to requests from various gastroenterology and pediatric programs around the country and internationally, we continue to analyze Crigler-Najjar Type I and II patients' DNA for defects at the *UGT1* locus. Recently, we carried out a prenatal diagnosis on DNA from amniotic cells to establish if an at-risk fetus carried a CN-I genotype. Initially, we demonstrated that the homozygous CT conversion mutation in the 3-year old sibling caused a loss of HUG-Br1 activity for glucuronidation. Since we are purifying the surrogate baculoviral egt protein for x-ray diffraction studies and then to derived its tertiary structure, the mutations uncovered in patients serve to distinguish conserved critical residues from conserved noncritical residues at the active site of the HUG-Br1 protein which will be used as guides in the computer-modeling of the data from the egt crystallin structure to derive an active site for HUG-Br1. These studies promise to provide the first estimation of an active site for a glucosyltransferase and a glucuronosyltransferase.

The *UGT1F* exon 1 (in position six at the *UGT1* locus) encodes the acetaminophen-selecting domain of the HLUG P1 isoform. We have uncovered two apparent missense mutations in one allele of the *UGT1F* gene of an individual, creating heterozygosity. The mutations are T181A and R184S affecting amino acid residues in the unique amino terminus of the isoform. We verified heterozygosity by restriction enzyme analysis of the genomic DNA of the individual. The defective cDNA has been inserted in an expression unit to generate protein in COS-1 cells as determined by immunocomplexing of radioactive HLUG P1 protein. We will now establish if activity is affected in the double mutant. Alternatively, we will establish if either mutation is a simple polymorphism. Since wide spread use of acetaminophen as an analgesic has uncovered cases of extreme sensitivity in the population, it has been postulated that defects occur in the enzyme involved in its detoxification. The equivalent, but defective, isoform in the Gunn rat that carries a mutation in a common exon at the *UGT1* locus is responsible for a 100-fold increase in toxicity and death following treatment with high doses of acetaminophen compared to the effect seen in the normal Wistar rat. The possibility exists that we have uncovered the first mutation in humans which is responsible for increased sensitivity to acetaminophen.

By using the UGT1A-upstream region-reporter gene fusion construct, we have shown that the reporter gene has liver-specific constitutive regulation and promoter strength comparable to that of the very strong SV40 promoter element which is used as a positive control. Further the constitutive activity is dependent upon sequences between -700 bp and -1700 kb upstream of *UGT1A*. Further analyses are continuing.

The bile acid, hyodeoxycholic acid, UDP-glucuronosyltransferase cDNA isolated and characterized in our laboratory continues to undergo analysis for expression in diseased and normal liver samples to determine if there is a relationship between the enzyme level and choleostatic liver disease.

We are collecting human prostate and testicular tissues from individuals from puberty to greater than 70 years of age to determine the pattern of mRNA encoding the dihydrotestosterone UDP-glucuronosyltransferase recently isolated in our laboratory. Abnormalities in dihydrotestosterone glucuronidation have been associated with benign prostatic hyperplasia (BPH) and prostate cancer as reported in the literature.

Significance to Biomedical Research and the Program of the Institute:

Drug oxygenations and subsequent glucuronidations are catalyzed by a endoplasmic reticulum-bound multistep enzyme complex. These enzymes are important in the process of detoxification and elimination of many normal body constituents and many foreign chemicals taken in from our environment in the form of food additives, air pollutants, and medication. There are, as well, diseases of humans ascribed to defects (not well understood) in drug-conjugating enzymes. Examples in pediatrics include neonatal hyperbilirubinemia, Gilbert's syndrome, and the Crigler-Najjar diseases. Bilirubin, one of the most toxic metabolites in the body undergoing constant production from the normal turnover of red blood cells, is completely dependent upon the UDP-glucuronosyltransferase for its detoxification. Unconjugated, the heme derivative is a potent neurotoxin which leads to jaundice and kernicteric (brain) injury.

Furthermore, there are drug interactions in which the administration of two or more drugs concurrently may alter the half-life and, thus, the pharmacological effect of one of the drugs. This type of alteration in drug half-life has often been ascribed to competition of substrates at the site of glucuronidation. The extension of a drug half-life can be critical when there is a narrow margin of safety. Idiopathic drug reactions are also attributed to differences in glucuronidation rates between patients; however, there is no verification that glucuronidation plays a role.

Teratogenesis and neonatal drug toxicities are proposed to occur as a consequence of deficient glucuronidation with critical drugs, e.g., dilantin and chloramphenicol. Further, impaired/absent dihydrotestosterone glucuronidation has been associated with benign prostatic hyperplasia (BPH)/prostate cancer, respectively.

The recent isolation and characterization of the bilirubin transferase gene are important to the detection of the nature of the defective allele(s) from the Crigler-Najjar Types I and II patients and from Gilbert's individuals whose alteration(s) is also inherited. With the isolation of the gene we will be able to compare replacement of bilirubin transferase activity in the Gunn rat model with the gene, as well as the cDNAs (also isolated in our laboratory). If we are successful in establishing a Crigler-Najjar cell line and restore

bilirubin transferase activity in Gunn rat, these replacement experiments can be repeated in the human mutant cells. Research which contributes to the improvement or elimination of the neurotoxicity of bilirubin and the associated emotional stress in youths caused by the jaundiced skin tones represent a primary goal of the Institute to improve the health of children. This work will also lead to therapies for defective bilirubin metabolism.

To the contrary, recent studies have demonstrated that the acyl-group of certain glucuronides of NSAIDS can migrate creating a reactive "leaving group" that can covalent attack endogenous molecules, particularly proteins, leading to haptenes and the development of autoimmune responses with lethality in some individuals. This would represent a toxification model for glucuronidation.

Potentially, we have the opportunity to identify and characterize new transferase isoforms; some promise to be toxifying (acyl-glucuronides) as well as detoxifying (ether glucuronides acetaminophen). The results from such studies will lead to new considerations in drug development with respect to potential toxins.

Research findings which identify the human transferase cDNA encoding the enzyme responsible for glucuronidating and maintaining optimal concentrations of the critical bile salt in humans constitute a major advancement. Bile acids are important in the emulsification of fat in the diet, but excess hyodeoxycholic acid in serum and in the bile is associated with cholestatic liver disease. Since we have shown that phenobarbital induces the mRNA encoding this isoform, this or similar agents can be tested as therapeutic agents to counter the progression of cholestasis. The demonstration that the transferase which glucuronidates the bile acid also glucuronidates estriols and 4-hydroxy catechol estrogens provides a basis for understanding possible interactions between estrogen metabolites and the bile acid.

The recent characterization of a human dihydrotestosterone transferase cDNA including its location in target tissue, testis and prostate, as well as liver provides a model for the determination of the relationship between BPH and other prostate hyperplastic diseases and failures in dihydrotestoserone glucuronidation.

Proposed Course:

To determine by using bilirubin transferase-specific cDNA probes and designated restriction enzyme sites if the RFLP technique can be used to show allelic differences in Crigler-Najjar patients compared to the normal controls, siblings, and parents whether such a rapid and a specific diagnosis for the range of genotypes for the disease can be established.

To adapt the SSCP method as a preliminary screen for defects in Crigler-Najjar patients and Gilbert's individuals to provide a more rapid determination of defects.

To sequence the region identified by the SSCP technique to define the specific alteration in the *UGT1* gene complex in individuals with the Crigler-Najjar, Type I and II diseases and the Gilbert's syndrome.

To develop a Gunn rat liver cell model for bilirubin transferase gene replacement.

To collaborate with investigators at the NIH on the adaptations of the *in vitro* models for *in vivo* replacement of the bilirubin transferase cDNA or gene.

To make selected mutations in the HUG-Br1 and HUG-Br2 cDNAs followed by expression in COS-1 cells and assesssing the effects on catalytic activity.

To use cDNA constructs encoding two other bilirubin-like transferases based on newly uncovered exons in the gene complex for expressing the respective proteins in cell culture to assess glucuronidating activity.

To establish the substrate specificity of new isoforms uncovered in the extension of the original locus.

To determine the effects of mutations in nonbilirubin UGT1 isoforms uncovered using a range of therapeutic and nontherapeutic chemicals.

To study in detail the regulation of mRNAs encoding the bilirubin-and bilirubin-like transferases and that of the hyodeoxycholate acid transferase - using poly(A)$^+$RNA isolated from variously treated monkeys.

To continue to study enhancer and promoter activity (with and without appropriate inducer compounds) for the 5' flanking regions of the two bilirubin transferase genes using the gene upstream fragment-reporter gene fusion construct for transfection into the human liver Hep.G2 cell line.

To determine if other substrates are glucuronidated by the bilirubin transferases.

To establish kinetic parameters for the different transferase isoforms.

To make synthetic transferase peptides from deduced nucleotide sequence data to develop antibodies to carry out tissue distribution and localizations of isozymes especially for the human bilirubin transferase isoforms.

To collaborate in analyzing surgical prostate specimens to determine the level of mRNA encoding the dihydrotestosterone-specific transferase cDNA and disease state of the tissue.

To purify the soluble baculoviral UDP-glucosyltransferase for crystallographic studies to use as a model for a computer derived-model of the conformation of the bilirubin UDP-glucuronosyltransferase.

Publications:

Babu SR, Lakshmi VM, Owens IS, Zenser TV, Davis BB. Human Liver Glucuronidation of Benzidine. Carcinogenesis 15: 2003-2007 (1994)

Ciotti, M, Yeatman, MT, Sokol, RJ, and Owens, IS. Altered coding for a strictly conserved Di-glycine in the major bilirubin UDP-glucuronosyltransferase of a Crigler-Najjar Type I patient. J Biol Chem 270:3284-3291 (1995)

Owens, IS and Ritter, JK. Gene structure at the human *UGT1* locus creates diversity in isozyme structure, substrate specificity, and regulation. Prog Nuc Acid Res Mol Biol 51:305-338 (1995)

Owens, IS, Ritter, JK, Yeatman, MT, Chen, F. The novel *UGT1* gene complex links bilirubin, xenobiotics, and therapeutic drug metabolism by encoding UDP-glucuronosyltransferase isozymes with a common carboxyl terminus. Proc Natl Acad Sci (USA). (In press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00404-13 HDB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell and Sulfur Metabolism in Fibroblasts of Genetic Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jean DeBrohun Butler	Senior Investigator	HDB, NICHD
Others:	Edward McIver	Biology Aid	HDB, NICHD

COOPERATING UNITS *(if any)* Laboratory of Developmental and Metabolic Neurology, NINCDS (Peter G. Pentchev); Laboratory of Cell Biology and Genetics, NIDDK (Mark Levine); Section on Developmental Genetics, Heritable Disorders Branch, NICHD (Anil B. Mukherjee); Laboratory of Cellular and Developmental Biology, NIDDK (Joan Blanchette-Mackie).

LAB/BRANCH

Heritable Disorders Branch

SECTION

Unit on Genetic Diseases Involving Cystine Storage

INSTITUTE AND LOCATION

NICHD, NIH, 10/10N308 Bethesda, MD 20892-1830.

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
   ☐ (a1) Minors
   ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Commercially obtained cystine binding protein (CBP) of E. coli has been purified by HPLC, yielding a pure cystine binding protein not similar to any protein in the data banks. This protein has been partially sequenced, an N-terminal peptide made and an antibody to this peptide obtained which reacts with the whole protein. The commercial preparation of CBP also contains a histidine binding protein which can be used to assay for histidine in the same way that CBP is used to assay tissues for cystine. CBP of E. coli is being cloned by two methods. One, by using an E. coli expression library and identifying the correct CBP producing colony with our antibody to CBP. Two, by making oligonucleotides of sections of the known N-terminal sequence of CBP and using them as primers to identify CBP in the E. coli library. By this later method, we have obtained a partial sequence of the DNA of CBP and are are continuing the work to obtain the complete sequence. This DNA sequence will then be cloned in a suitable bacterium for large production of cystine binding protein. With this larger amount of material, the protein will be studied as to its metabolic function, specifically its cystine binding capability.

2. Niemann-Pick C disease (NPC) was shown to be reversibly induced in normal fibroblasts by hydrophobic amines and certain steroids, thus creating a model system for study of this disease. We are identifying the trafficking patterns and metabolism of intracellular cholesterol using various inhibitors and stimulators.

3. We have shown that normal and cystinotic fibroblasts take up ascorbic acid by two mechanisms, a high affinity Na^+ dependent transporter and a low affinity Na^+ independent transporter, neither of which can be accounted for by diffusion. Transport of ascorbic acid by the low affinity transporter is not different from normal in the two types of cells, however transport in cystinotic cells by the high affinity transporter is about 40% of that seen in normal cells. The Km's for both transporters were the same for both types of cells while the Vmax of the high affinity transporter was statistically lower than normal for cystinotic fibroblasts. This data indicates that cystinotic fibroblasts have a selective impairment in ascorbate transport.

Project Description

Objectives:

1. To elucidate the basic biochemical defect in genetic diseases, specifically cystinosis and Niemann-Pick C & D.

2. To explore secondary biochemical and physiological derangements caused by the primary metabolic defect.

3. To develop new and improved methods of treatment and diagnosis.

4. To use genetically abnormal cells along with normal cells to understand human basic biochemistry.

Methods Employed:

1. Cell tissue culture techniques.

2. Spectrophotometric, radiochemical, and fluorometric enzyme assays.

3. Ultracentrifugation and cell fractionation techniques.

4. Radioisotope measurements

5. Light and phase contrast microscopy.

6. Column, thin layer and high performance liquid chromatography.

7. Gel and high voltage electrophoresis.

8. Immunochemical techniques.

9. Molecular Biology techniques.

Major Findings:

1. Commercially obtained cystine binding protein of E. coli is heterogeneous. Purification gave four proteins. One protein bound cystine and was not similar to known proteins in data banks. Another protein was highly similar to a known histidine binding protein of S. typhimurium and can be used to specifically analyze tissues for histidine content as is now done for cystine.

2. Normal fibroblasts can reversibly be induced by hydrophobic amines and certain steroids to mimic NPC disease. With this model we are studying the development of this disease and subsequent restoration to normal using known agonists and antagonists of intracellular lipid transport and metabolism. We find that trafficking of intracellular cholesterol using both biochemical and cytochemical methods is via the golgi.

3. Normal and cystinotic fibroblasts have two ascorbic acid transporters, a high affinity Na^+ dependent transporter and a low affinity Na^+ independent transporter, neither of which are due to diffusion. One of these transporters is quantitatively different in cystinotic cells.

4. A mutant mouse was found which stores cystine and cholesterol in lysosomes of various tissues. This mouse is considered a model for NPC disease and now possibly for cystinosis. No animal model for cystinosis has previously been known.

Significance to Biomedical Research and the Program of the Institute:

The study of genetic inborn errors is a powerful tool for elucidation of biochemical mechanisms in normal as well as abnormal states. The discovery of formerly unknown metabolic pathways in cystinosis and in NPC disease in sulfur and in cholesterol metabolism will undoubtedly contribute greatly to our understanding of these processses in general and will lead to better methods of diagnosis, treatment, prevention and understanding of these and other disorders, and will have substantial public health significance.

Proposed Course:

Characterization of a cystinotic cystine associated protein in fibroblasts will be continued. CBP studies will be continued with the aim of finding the actual binding site for cystine in the purified CBP. Using immunochemistry and molecular biology techniques, the pure CBP of E. Coli will be cloned and proteins from eukaryotic cells will be sought with similar cystine binding properties and sites with the hope that a lysosomal cystine binding protein functioning in the egress of cystine from lysosomes will be identified.

All of the mutant diseases being studied where cystine and/or cholesterol are stored intracellularly (NPC model mutant mouse, cystinosis, I-cell disease and NPC), show a clinical degenerative neurological involvement. Cholesterol is absolutely essential for cell viability and the cellular level of this sterol is closely regulated and finely tuned. Abnormal changes in cellular cholesterol levels can have devastating effects neurologically and excess membrane cholesterol is known to inhibit transport mechanisms. The pathway for cholesterol homeostasis has been brilliantly worked out by Brown and Goldstein and is used as a model system for receptor mediated cellular uptake. However, we are finding in working with the above mutant cells that in the case of cholesterol homeostasis, additional previously unknown controls must be operating, particularly in the area of transport of cholesterol out of the lysosome to its sites of cellular regulatory responses. It has been assumed that cholesterol diffuses or is carried by a protein from lysosomes to processsing sites. NPC cells do not display this movement of cholesterol, rather free cholesterol accumulates in the lysosomal and golgi regions. We are testing these cells for lack of a normal cholesterol transport system or for the presence of an abnormal cholesterol binding system. Discovery of a way to create the NPC disease state in normal fibroblasts is very advantageous to these studies of the trafficking patterns of cholesterol in fibroblasts. The mutant mouse and I-cell disease where cystine and cholesterol are stored in lysosomes and cystinotic fibroblasts where cystine is stored in lysosomes will be similarly tested to ascertain their cholesterol regulating mechanisms. In cystinotic fibroblasts where cystine accumulates in lysosomes, there is no simultaneous accumulation of free cholesterol, however intracellular levels of stored excess cholesterol esters are low and circulating cholesterol levels in patients are high. This may reflect an abnormal cellular handling of cholesterol by cystinotic cells.

We are continuing studies on the kinetics of uptake of ascorbic acid (AA) by normal and cystinotic fibroblasts showing two transporters. Ascorbic acid lowers the cystine content of cystinotic fibroblasts

by unknown mechanisms. The knowledge that the Vmax of uptake of ascorbic acid by cystinotic cells is normal for the low affinity transporter and deficient for the high affinity transporter, indicates a need to explore the intracellular use of this vitamin in these mutant cells.

We are also investigating alternative ways of depleting cystine and/or cholesterol in fibroblasts and in mouse tissues since there has not previously been an animal model for the above diseases.

Publications:

Bailey JM, Bright R, Tomar R and Butler J. Anti-Atherogenic Effects of Cholesterol Vacination. Biochem. Soc. Trans. 22: 433S, 1994.

Blanchette-Mackie EJ, Coxey RA, Butler JD, Roff CF, Campbell G, Dwwyer NK, Carstea ED, Goldin E, Dawidowicz EA, Brady RO and Pentchev PG. The Intracellular Distribution of Cholesterol can be Modulated at Lysosomes, the Golgi Complex and the Rough Endoplasmic Reticulum. Proc. Nat. Acad. Sci. (In Press).

Ulane MM, Butler JDeB, Peri A, Miele L, Ulane RE and Hubbard VS. Cystic Fibrosis and Phosophatidylcholine Biosynthesis. Clinica Chimica Acta 230: 109-116, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00408-12 HDB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Heritable Disorders of Connective Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joan C. Marini, M.D., Ph.D.	Senior Investigator	HDB, NICHD
Others:	Qin Wang, M.D.	Fogarty Fellow	HDB, NICHD
	Antonella Forlino, Ph.D.	Fogarty Fellow	HDB, NICHD
	Piotr Wieczorek, Ph.D.	Fogarty Fellow	HDB, NICHD
	Anna Gajko, Ph.D.	Special Volunteer	HDB, NICHD
	Diane Trybul	Biologist	Ebon Contract

COOPERATING UNITS *(if any)*

Department of Rehabilitation Medicine, CC (N.L. Gerber); Laboratory of Developmental Endocrinology (G. Chrousos); Sect on Metabolic Analysis and Mass Spectrometry (A. Yergey); Genetics Unit, Shriners' Hospital, Montreal (F. Glorieux)

LAB/BRANCH

Heritable Disorders Branch

SECTION

Connective Tissue Disorders

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.25	3.5	.75

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Section has continued its studies aimed at elucidating the molecular mechanisms of heritable disorders of connective tissue, specifically osteogenesis imperfecta (OI) and Ehlers-Danlos (EDS), and at applying this information to the treatment of these disorders.

One primary interest of the Section is to identify the collagen mutations in patients with OI and EDS and determine the relationship between the type and location of the mutation and the severity of the connective tissue disorder. Mutations in the $\alpha2(I)$ collagen chain identified by this Section and other labs have provided additional support for the regional model we have proposed.

A second primary interest of the Section is to develop selective antisense suppression of the mutant collagen allele as an approach for therapeutic intervention. We have used thio oligos targeted to mutant mRNA and nuclear RNA in the cultured fibroblasts of a patient with type IV OI. More recently, we have successfully used ribozymes *in vitro* to obtain allele-specific cleavage and are now pursuing vector constructs to increase suppression stability and efficiency.

A third focus of interest which we have been developing is in the bone biology of OI. We are using cultured osteoblasts to study the way bone cells modify and secrete mutant collagen. We are also pursing the secondary non-collagenous abnormalities of OI matrix.

In clinical studies, we are continuing our treatment trial of growth hormone in short children with OI to determine its effects on growth stimulation, bone density and bone morphometric properties. We are continuing our collaborative interests in the neurological aspects of OI and in maximizing the physical functioning of OI children though aggressive rehabilitation.

Project Description

Objectives:

To study the molecular biology and pathophysiology of genetic disorders of connective tissue and to use the findings of these studies for diagnostic and therapeutic intervention.

Methods Employed:

Culture of human fibroblasts, osteoblasts and chorionic villi; selective labelling of collagens and proteoglycans; standard methods of protein purification; protein analysis by SDS gels, gradient gels, cyanogen bromide cleavage of peptides; methods of molecular biology such as preparation of plasmids, subcloning, restriction enzyme mapping, riboprobe generation, coupled transcription/translation, isolation of total RNA, RNase cleavage of RNA/RNA and DNA/RNA hybrids, sequencing, cDNA screening, amplification by polymerase chain reaction (PCR) of genomic and cDNA, single strand conformational polymorphism analysis (SSCP), allele specific hybridization, antisense suppression, Southern and Northern hybridizations.

Major Findings:

1) As an approach for therapeutic intervention in OI and other dominant negative disorders, we are investigating the effectiveness and selectivity of antisense suppression of the mutant collagen chain using lipid-mediated transfection. We have turned our focus to the use of antisense hammerhead ribozymes to increase both specificity and efficiency. For these *in vitro* experiments, we are using the $\alpha 1$(I) alleles from a family with a unique collagen mutation. This mutation was previously described in our lab and consists of a gene level deletion extending from exon 34 to about the middle of intron 36. The 3'-end of intron 36 is retained in the mutant message and the sequences are in frame and contain no stop codons. This allows us to target hammerhead ribozymes to exon 35 in the normal allele and intron 36 in the mutant allele.

We first investigated the *in vitro* cleavage of each mRNA by its specific ribozyme. For this purpose, we subcloned the aa579-679 portion of each allele containing the target region in pGEM vector. This allows us to generate labelled synthetic RNA target. When this RNA synthetic sense strand is mixed with the appropriate ribozyme and Mg, specific cleavage occurs at precisely the predicted location. The amount of cleavage increases as the ratio of RZ:target increases, with minimal cleavage at a 1:1 ratio and maximal cleavage at 50:1. Cleavage also increases with time, converting virtually all target to product in 2 hrs and confirming the catalytic nature of the ribozymes. Finally, we confirmed the allele specificity of the ribozymes by incubating each ribozyme with sense and antisesne target from each allele; for each ribozyme, cleavage was obtained only with the sense strand of the matching allele. The crucial negative was that the mutant ribozyme does not cleave the sense strand of the normal allele.

To begin suppression experiments in cells, we have investigated the lipid-mediated transfection of ribozyme into the cells. The percent of RZ taken up by the cell was greater with lower concentrations but total uptake was highest with 1.0 uM RZ and 20 ug/ml lipid. The higher concentration of lipid, however, decreased cell survival. As a compromise we have done transfections with 0.4 uM RZ and 15 ug/ml lipofectamine. No suppression of the mutant message was detected; vector mediated transfection may be required to achieve practical intracellular levels of ribozyme.

We have also begun to investigate the use of circular oligonucleotides for antisense suppression. The resulting triplex structure would have increased binding stability as compared to a linear oligonucleotide.

We labelled this oligo for transfection experiments using tritiated water and the hydrogen-tritium exchange at the purine C8 position. Uptake of circular oligos was slower than for linear molecules, with no appreciable transfection until t=1 hr. The maximum uptake of circular oligo at 6 hrs post transfection, about 15%, was lower than with linear oligos and was achieved with oligo at 0.4 uM and lipid at 15 ug/ml.

2) We have previously identified a unique deletion/insertion collagen mutation in a family with dominant type III OI and a pedigree suggestive of germline mosaicism. The cDNA from one α1(I) allele has a deletion of the sequences coding for exons 35 and 36 (aa 604-639), followed by an insertion of 156 nt from the 3'-end of intron 36. At the gene there is a 565 nt deletion whose ends are identical to those of the cDNA deletion. We have now examined the genomic DNA from WBC of the members of the extended family. Presence of the mutation is detected by genomic PCR using a primer set which generates a 1007 bp fragment from the normal allele and a 445 bp fragment from the mutant allele. The paternal grandmother was previously demonstrated to be the mosaic carrier in the family. We have now shown that the three clinically normal daughters of the mosaic carrier have only the normal allele.

Although the cDNA intronic insertion is in frame and contains no stop codons, we had been unable to detect the mutant protein. To demonstrate that the mutant protein was in fact translatable, we subcloned the collagen cDNA coding for aa 430-732 into pGEM3Z vector, using primers to add a start signal to the 5'-end and a poly A tail and three stop codons to the 3'-end. Using the TNT T7 reticulocyte coupled transcription-translation system for in vitro translation, we confirmed that the mutant protein is translatable. The product of the expected size, 35 KD, was generated.

In order to examine OI fibroblast and bone cells for the presence of low levels of the mutant type I collagen containing a non-collagenous hinge, we are utilizing antibodies specific for the sequences encoded by the retained intronic sequences. A 20 aa peptide was synthesized from the intronic sequences and used to generate polyclonal antibodies. We are now doing immunoprecipitations using this antibody and the *in vitro* translated mutant protein, with the goal of demonstrating that the antibody will detect the mutant protein specifically.

3) We have continued to investigate the collagen protein abnormalities and associated molecular defects in patients with osteogenesis imperfecta. We have previously proposed a Regional Model for the relationship between clinical severity and mutation location. This model is based on the clustering of lethal and non-lethal phenotypes along the α2(I) chain, which suggests that the location of the mutation is the most important factor in phenotype determination for single amino acid substitutions and single exon splicing defects. The new mutations which have been delineated along the α2(I) chain are compatible in location with the regional model, in which there are now 3 lethal and 4 non-lethal regions alternating along the length of the chain.

4) One important consequence of the regional model is that it delineates which regions of the chain are crucial for collagen/extracellular matrix structure and allows further research to focus on determining the specific role(s) of the crucial regions. One possible role for crucial regions would be to engage in collagenous or non-collagenous interactions in matrix.

We have begun to purify type I collagen from several OI cases in each of the regions along the α2(I) chain for the purpose of investigating possible abnormalities in binding functions. We have now purifed preparative quantities of type I collagen from 5 OI cases and normal controls. Patient collagen is ammonium sulfate precipitated from the media of continuous cultures of fibroblasts after ascorbate stimulation. DEAE-cellulose 52 chromatography is utilized to remove proteoglycans. A second DEAE-cellulose 52 column is eluted with a 0-0.12 M NaCl gradient. This allows the reasonable separation of ColI pC forms, type I procollagen, type III procollagen and fibronectin. Appropriate fractions can be pooled and concentrated for further studies.

5) We have continued to study germline mosaicism in our patients. We are investigating this mechanism of inheritance of OI in both lethal and non-lethal forms of OI.

We are investigating four families with non-lethal OI and presumptive evidence of mosaicism. The first family has the α1(I) deletion/insertion mutation described in item #2, above. In the second family with non-lethal OI, the mother had an OI child with each of two different spouses. We have previously demonstrated that the mutation in this case is an α1(I) gly 688 → ser and that the clinically normal mother has a M:N::48:52, showing high expression at the leukocyte level. In a third non-lethal family, the CB6 peptide migrates slightly fast. SSCP analysis of subfragments in this region suggested that the mutation might be between aa 843-889. To examine the α2(I) cDNA, we subcloned a fragment encoding aa 759 to C-terminal and used a common C → T polymorphism at nt 3270 to distinguish the alleles. Both alleles were sequenced in the entire subcloned region but no mutation was detected. To examine the α1(I) chain, we checked for heterozygosity of common polymorphisms in the C-terminal quarter of the helix. No polymorphisms were found and the α1(I) cDNA remains to be sequenced. In family 4, analysis of collagen synthesized by skin fibroblasts of the clincially normal mother and the two affected sons showed delayed migration. The α2(I) alleles of the patient were distingished using a known polymorphism. Both alleles were sequenced for the region aa 226-323 but only polymorphisms were detected.

We are investigating three families with lethal OI and presumptive evidence of germline mosaicism. We have demonstrated in two of these cases of recurrence of lethal OI to clinically normal parents that all cyanogen bromide peptides are overmodified and that the mutations must reside in the carboxyl 20% of the helix. Both cases were screened by SSCP of this region of α1(I) and α2(I), but no mutation was detected. We were able to separate the α2(I) alleles in both families, using heterozygosity for a common A→C polymorphism at Pro392. The α2(I) alleles were sequenced for the region encoding aa 550 to the end of the helical region in one family and from aa 550 to the COOH-end of the peptide in the other family. No mutations were detected and we have now turned our focus to α1(I) in these families. The third case is a very interesting family in which each parent has had a normal child with another partner but together they have now had three children with the lethal form of OI and no normal children. Our examination of offspring collagen indicated that overmodification began amino-terminal to α1(I) CB6. RNA hybrid analysis has yielded a candidate region of α1(I) aa 558-861.

6) We are continuing our interest in the molecular basis of variability of expression of the same collagen mutation. We have delineated the same α1(I) mutation, gly 352 → ser, in two families. In one family, a man with severe type III OI has two sons affected with moderate type IV OI. In an unrelated family, the mutation occurs in a girl with type III OI. Subsequently, this mutation was identified in an OI type III cell line in a cell bank. The genetic phenomenon of variable expression occurs particularly in dominant structural disorders. Of the small number of recurrences of collagen mutations which have been delineated to date, this represents the only known instance in which individuals with the same collagen mutation have dramatically different phenotypes and is thus an especially appropriate situation for further study. We have begun to examine this question by sequencing the α1(I) cDNAs for secondary mutations. In all five affected individuals, we have sequenced the region coding for α1(I) aa 1-558 in both normal and mutant alleles and found no secondary mutations. One non-conservative change (Pro → Ser at aa 71) was detected in both alleles of all five individuals. We are now subcloning and sequencing the COOH-half of the α1(I) cDNA from the affected individuals. In addition, we wish to investigate whether the variable phenotype is associated with differences in the level of expression of mutant and normal α1(I) alleles. For this purpose we have set up an ASO system using 17-mer oligos with a central mismatched nucleotide. These probes have been demonstrated to be allele specific at the Southern blot level.

We are also investigating a second pair of unrelated OI cases with the same mutation (α2(I) gly 922 → ser). We have described one of the two cases and the second case was identified by colleagues in Italy. In contrast to the variable expression described above, these cases have essentially the same severity of type

IV OI. We have shared our patient's fibroblasts with our Italian colleagues; studies comparing the collagen deposited in matrix in the two cases yield similar results.

7) We are continuing our investigation of osteoblasts cultured from the iliac crest biopsies or osteotomy chips of OI patients. Our goal is to gain insight into the molecular and biochemical mechanisms underlying bone pathophysiology in OI. We have performed pulse-chase experiments on 5 cases of cultured OI osteoblasts and fibroblasts from the same patient as well as on control lines of osteoblasts and fibroblasts. Cells were plated as usual and labelled with ^{14}C-proline for 2 hrs; then the media was removed and replaced with a chase media containing 2.0 mM unlabelled proline. The secretion of the labelled collagen over the following 6 hrs was followed by densitometric analysis of SDS-PAGE. In general, osteoblasts secrete collagen more rapidly than fibroblasts. About 50% of pulsed collagen is secreted in the first 0.5 hrs of the chase, whereas fibroblasts require about 2 hrs to secrete half the pulsed collagen. In one of the OI cases, the mutant and normal collagen chains were secreted from fibroblasts at approximately equal rates. In the osteoblasts, the mutant chain was secreted at half the rate of the normal chain.

We have also continued to expand our supply of frozen OI osteoblast lines. This year we have cultured osteoblasts from 10 patients with types III or IV OI.

8) We have initiated a new project in co-operation with the Laboratory of Dr. Heiner Westphal at the NICHD for the generation of a conditional non-lethal murine model for OI. The general plan involves (1) site-specific mutagenesis of a glycine in a region of α1(I) which is known to be non-lethal in humans; (2) insertion of a lox-stop-lox signal into the intron proceeding the mutation in order to stop transcription and translation of the mutant COL1A1 gene and to make expression conditional upon the presence of a cre recombinase; (3) insertion into the germ line using ES cells and homologous recombination, in order to have the mutant construct under the control of the endogenous promotor.

We obtained a plasmid containing exons 8 to 40 of the COL1A1 gene from the mouse strain 123/SV from Dr. R. Jaenish. We generated a restriction map of this fragment and subcloned a 10 KB EcoRI-EcoRI segment of DNA containing the exon 22-23 region into pBluescriptII. This fragment was mapped in detail for restriction sites for 17 enzymes. For site-specific mutagenesis, a 4.2 KB XbaI-KpnI region was further subcloned. We plan to insert four point mutations, changing gly 343 to cys as the disease causing mutation, leu 356 to met to provide dectability of the mutant protein chain by cyanogen bromide digestion, and changing the codon for arg 344 to create an allele-specific ribozyme cleavage site. For insertion of the lox-stop-lox signal, we have sequenced the murine intron 22, whose sequence was previously unknown, and have selected a restriction site for insertion of the stop construct.

9) We have continued to screen the type I collagen of OI patients both as a genetic service to families and to identify cases of particular research interest. The type I collagen of 12 probands and their families was analyzed this year. Of particular interest have been the fibroblast collagens of patients from whom we are also studying the cultured osteoblasts. These fibroblasts are of interest not only for determination of the underlying mutation but also for comparison of expression and secretory patterns between different cell types. No CVS samples have been screened this year.

10) We are continuing our studies of Ehlers-Danlos Syndrome (EDS), a connective tissue disorder whose symptoms overlap those of OI but do not include brittle bones. We are currently investigating nine cases of EDS.

In four of these cases, we find the same delay of electrophoretic migration of type I collagen chains and specific CNBr peptides, as is routinely found in OI. In three of these cases, unmodified collagen chains also have abnormal gel electrophoretic migration, suggesting the possibility of substitutions for non-glycine residues.

In one EDS case, the type I collagen chains are more rapidly migrating than normal. All the CB peptides are also rapidly migrating suggesting generally decreased modification. We are collaborating with the laboratory of Dr. Heather Yeowell at Duke University. She has demonstrated decreased activity of lysyl hydroxylase in the fibroblasts of these patients. We have now supplied her with cultured cells from this patient and are awaiting PCR analysis of the lysyl hydroxylase of this patient with EDS type VI.

We are investigating 4 cases of presumptive type IV EDS, by clinical criteria. We have utilized the technique of delayed reduction of type III collagen on SDS-Urea-PAGE to demonstrate electrophoretic abnormalities in two of these cases. In one case there is a greater than 80% reduction in the quantity of type III collagen, the remaining collagen III has normal elecrophoretic migration. In the second case, there is the unusual situation that the amount of type III collagen is essentially normal, but it has more rapid elecrophoretic mobility. Column chromatography did not yield sufficient type III collagen for cyanogen bromide analysis on unlabelled type III chains from this second proband.

11) We have been engaged in a collaborative project with our consulting orthopedic surgeon, Dr. C.M. Reing, and the Smith and Nephew Richards, Co. for the development and testing of alternative rodding materials for children with osteogenesis imperfecta. The Richards Co. has now been able to formulate resorbable rods with the desired breakdown time and we are ready to proceed with Phase II of the project.

12) We have continued our clinical protocol for the Growth Hormone Treatment Trial in Osteogenesis Imperfecta. To date, 20 children have completed a year of treatment using recombinant growth hormone from Genentech, Inc. A total of 11 children have experienced a 50% or greater increase in linear growth rate, and are continuing to respond positively during the 2nd 12 month phase. For all children treated, there is a highly significant rise in IGF-I and IGFBP-3 values from baseline to 1 year, but these values did not distinguish responders from non-responders. At one year of treatment, changes in IGFBP-2 and the L2-L4 vertebral bone density distingish responders and non-responders. There is also a positive change in bone morphometric studies pre vs post treatment: the bone volume and the mineralized bone volume per tissue are both increased.

13) We continued our clinical trial, in collaboration with Dr. Lynn Gerber of the Clinical Center Rehabilitation Medicine Department, of the effects of active rehabilitation and lower limb bracing on children with moderate and severe OI. The cross-over study of weaning OI children from braces was completed. The parameters evaluated include muscle strength, fracture rate and activity. There was more decline in muscle strength in the unbraced period. Levels of strenuous activity were greater during the braced period. There was no statisitically significant difference in fracture rate during braced and unbraced phases of this trial. Several of the the OI children were able to be weaned without increase in fracture number. We are continuing to collaborate with Dr. Young Jack Lee in the analysis of these data.

14) We have continued our interest in the neurological aspects of OI, in collaboration with Dr. Lawrence Charnas. The clinically significant neurological finding is basilar invagination and consequent brainstem compression. This secondary feature of OI and the potential seriousness of its consequences, was unappreciated prior to this collaboration. Our interests include the delineation of the natural history of BI and determining the optimum nature and timing of the surgical response. Our patient population currently includes 11 children with documented basilar invagination, 7 of whom have brainstem compression. Progressive compression of brainstem and symptomatic extension of syrinx in one teenage patient necessitated a cervical fusion to the occiput. Short term results of this procedure have been good. We have begun to screen candidates for BI with both spiral CT and sagittal MRI, to determine the more sensitive test for detecting and following the progression of BI.

15) We have continued our collaboration with Dr. A. Yergey for the analysis of calcium kinetics in children with OI. Six children have participated in this study in this past year; 12 of the 20 children in the growth hormone treatment trial have had before and after treatment calcium kinetics. Preliminary results

of stable isotopic calcium metabolism/distribution studies in Types III and IV OI, showed an increased rate of calcium accretion to bone after treatment (type III OI, 94 ± 29 mg/kg/d pretreatment vs 142 ± 75 mg/kg/d post treatment, $p \geq .1$ and type IV OI, .64 ± 14 mg/kg/d pretreatment vs 91 ± 19 mg/kg/d post treatment, $p = 0.012$). While suggestive of possibly important consequences, the numbers will be reanalyzed with the six additional studies done this past year.

Significance to Biomedical Research and Program of the Institute:

This project attempts to elucidate the molecular biology of several genetic diseases of connective tissue at the gene, cell and tissue levels and to apply these findings to the treatment of the disorder.

We are interested in determining the relationship between the type and location of mutations in types I and III collagen and the pathophysiology of OI and EDS. These studies have broad implications for our understanding of skeletal biology and the interactions of the components of matrix. The study of OI mutations has led us to a regional model which suggests that some regions of the chains have an absolutely crucial role in higher order structures; we can now do binding experiments to try to determine the interactions for which the lethal regions are crucial. In addition, as dominant disorders of structural proteins, these disorders are excellent model systems in which to study the general genetic phenomena of variability of expression and parental mosaicism.

Because of the role of collagen as a structural, rather than an enzymatic protein, future gene therapy of these disorders will require that the defective allele be selectively turned off. We have been using transient lipid-mediated antisense suppression of the mutant allele in cultured fibroblasts as a first step in discriminative targeting. Development of this novel form of gene therapy may include modifications to enhance selectivity, vector mediated stable transduction, and targeting of osteoblasts. Development of a mouse model for moderately severe OI would allow testing of the new treatment modality in marrow stem cells.

We have recently turned our attention to the bony matrix in OI by initiating studies of cultured OI osteoblasts. The primary defect of collagen has secondary consequences for the non-collagenous proteins of bone and for the interactions of osteoblasts with matrix, resulting in a globally abnormal matrix. Studies of the structure and control of expression of osteoblast products in OI verses normal cells will enable us to understand how the collagen mutations are translated into a defective skeletal phenotype. This insight into osteoblast and matrix biology has broad implications for the management of other disorders of matrix or mineralization.

Our clinical protocols are conducted for the most part with the same patients whose collagen is studied in the laboratory, enabling us to correlate patient's clinical response, bony matrix characteristics and primary collagen defect. Our interest in growth characteristics of abnormal matrix is reflected in our growth hormone treatment trial, testing the effect of hormone on linear growth and on matrix quality, its mineralization, cellular and trabecular structure. We plan to enlarge our growth studies to examine the plateau phase of growth in OI (ages 1-4 yrs) and the effects of puberty. We continue to lead a multidisciplinary effort in aggressive physical rehabilitation of children with OI to maximize functional level.

Proposed Course:

1) We plan to continue our development of selective antisense suppression of mutant collagen as an approach to therapeutic intervention in OI. We will determine whether the *in vivo* selectivity of antisense

ribozymes is comparable to the high *in vitro* selectivity. We plan to compare their effectiveness against retained introns, altered exon junctions and point mutations. We will also compare the effectiveness of ribozymes to linear oligos and circular oligonucleotides. For all these situations, we will examine the effect of the suppression at both the protein and mRNA levels.

We are also interested in comparing the selectivity and effectiveness of antisense oligos in osteoblasts in culture. As these cells are less accessible, we would plan to use osteoblasts from an OI patient with a cysteine substitution and several of the most effective suppression structure, as determined in fibroblasts.

Both antisense oligos and ribozymes could be produced with high intracellular expression and with stable gene delivery by the use of a retroviral construct. There are currently available two such vectors, which use pol III tRNA promotors and termination signals to achieve high transcription levels and expression in a wide range of tissues. Since retroviral vectors are still preferred for stable delivery of genes to hematopoietic cells, these constructs would be suitable for transfer to stem cells. We would plan to test the *in vivo* effectiveness of such constructs in tissue culture and, eventually, in an appropriately engineered transgenic mouse.

2) We will continue to develop a murine model for non-lethal OI in collaboration with Dr. Heiner Westphal's lab. The primary purpose of such a murine model would be as a testing subject for selective antisense therapy. Additional purposes for such a model would be to provide a continuous supply of osteoblasts for study and for the testing of potential drug regimens. We plan to use the cre/lox recombinant system of bacteriophage P1 to create a conditional mutation. We will first complete construct generation, which comprises both site specific mutagenesis in exon 23 of the α1(I) subclone and also creation and insertion of the lox-stop-lox signal in intron 22. The mutant allele construct will be returned to the 8 KB murine construct for ES cell injection and homologous crossover.

3) For the novel deletion/insertion mutation in α1(I) which we have identified, we plan to investigate the possibility that alternative splicing of the retained intron occurs. If so, we will investigate the possibility that the proportion of alternative forms of the mutant protein correlates with the variations of phenotype in this family with type III OI.

4) We will expand our investigations of the cultured osteoblasts from patients with OI. The goal of these investigations is to correlate the molecular defects of collagen with biochemical and molecular abnormalities of osteoblasts in order to understand how the mutations cause a range of phenotypic abnormalities.

First, we will follow up on our observation that some OI osteoblast collagen displays different gel electrophoretic migration than fibroblast collagen from the same patient while other OI fibroblasts and osteoblasts synthesize collagen with the same electrophoretic abnormality. We are completing the identification of the causative collagen mutations in each of these OI cases. This will allow us to determine if there is a correlation of mutation location, in a particular chain, for example, and the behavior of osteoblast collagen. Our current hypothesis is that the osteoblast is more "tolerant" of mutant collagen than the fibroblast, resulting in less intracellular degradation and more secretion of the mutant helices. To test this hypothesis, we will compare degredation of the mutant chain in fibroblasts and ostoblasts. We are also collaborating with Dr. David Eyre of Seattle, who will examine by peptide sequencing the collagen protein secreted into the matrix by fibroblasts and osteoblasts from a child with an α1(I) gly 832 → ser mutation.

We also plan to investigate the non-collagenous products of the OI osteoblasts and relate their quantity and structure to the primary collagen abnormality. We plan to use Northern blots, Western blots, and antibody precipitations. We will study the synthesis, secretion and gel electrophoretic mobility of the proteoglycans biglycan and decorin, the bone specific proteins osteocalcin and BSP, and other bone proteins such as

osteopontin and osteonectin.

We are also interested in the ability of the OI osteoblast to respond properly to exogenous growth factors and to synthesize and secrete growth factors. We have been collecting conditioned media from our cultured OI osteoblasts. We will use ligand blots with labelled IGF to test for the presence of IGF-I binding proteins. As quantities of cultured osteoblasts allow, and guided by the results from the conditioned media, we would investigate the quantity and responsiveness of receptors for GH, or IGF-I or TGF-β.

We also plan to investigate the responses of the OI osteoblast to growth factors. We will treat cultured osteoblasts with GH, IGF-I and TGF-β and investigate the cell response in terms of proliferation, and the synthesis and structure of collagenous and non-collagenous products. In addition, TGF-β has been shown to increase the amount of secreted collagen which is cell associated. We will test whether the OI osteoblasts and the abnormal collagen can respond properly to this signal.

5) We will continue our molecular investigations of germ line mosaicism, focusing on families in which clinically normal parents have more than one child with a dominant form of OI. We will define the causative mutations in the affected offspring, then use WBC genomic DNA to determine which parent is the carrier. We will then explore expression of the mutant allele in a variety of tissues of the carrier. In our family with an α1(I) deletion/insertion, we have identified the paternal grandmother as the mosaic carrier. When she died recently of an unrelated stroke, the family made a complete autopsy available. This will provide very valuable and indeed, unique, scientific data. We will be able to study the expression of mosaicism in virtually all organs and tissues using *in situ* PCR of paraffin imbedded sections. Because germ line mosaicism appears to be present in up to 10% of clinically normal couples who have had a single OI child, development of timely prenatal screening is an important clinical as well as genetic problem.

6) We will continue to delineate the molecular defects in the type I collagen of osteogenesis imperfecta patients using a combination of the RNA hybrid techniques developed in this lab and SSCP. We plan to add CSGE (Conformation Sensitive Gel Electrophoresis) to our detection systems. Delineation of additional natural mutations is valuable because (1) this will allow us to further define the boundaries of the crucial and non-crucial regions in our regional model of OI pathophysiology; (2) knowledge of point mutations is essential information for studies on variable expression, mosaicism, antisense suppression, osteoblast metabolism and correlations with clinical data. We now have fibroblasts in storage from 24 cases in which we know the CNBr peptide pattern and from 56 additional cases in which electrophoretic abnormalities of the collagen chains have been demonstrated. Cases in which osteoblast samples are available for correlation or the family genetic pattern is particularly interesting will also be prioritized.

We will also continue our collagen protein screening on OI fibroblasts and CVS as a genetic service to families and as a source of new cases.

7) We plan to use the mutant type I collagens which we will purify for binding studies to investigate the possible functions of the "crucial" regions of the collagen chains identified in the regional model. One possible role for crucial regions would be to engage in collagenous or non-collagenous interactions. We have a collaborative agreement with Dr. Robert Spiro of La Jolla for the first of these binding investigations. We will provide Dr. Spiro with mutant type I collagen which he will investigate in his gel retardation assay for binding to decorin.

To investigate collagen interactions with other non-collagenous proteins, the target collagen, in either intact helical form or in heat denatured form, would be used to coat polystyrene microtiter wells. Collagen-coated wells would be exposed to a range of concentrations of normal type I, III and V collagen, osteonectin, osteocalcin (bone gla protein), osteopontin (BSP I), BSP II, and decorin (PG II). Dependence of binding of BSPs on calcium would also be investigated. Binding data would be subjected to Scatchard

analysis.

8) We plan to pursue our studies on the molecular basis of variability of expression, concentrating on the α1(I) gly 352 → ser mutation. We will consider collagenous as well as non-collagenous effects. For collagen, we will consider both structure and expression. We will complete the sequencing of the α1(I) and α2(I) cDNA of the genetically relevant individuals to look for secondary mutations. For collagen expression, we will determine the relative levels of the mutant and normal α1(I) mRNA and the α2(I) mRNA at the fibroblast level with allele specific probes. We will also perform pulse chase studies to compare the efficiency of secretion of collagen from fibroblasts.

We also have cultured and frozen osteoblasts from two of the affected individuals. We will obtain mRNA from cultured osteoblasts and compare relative levels of expression of the mRNAs and secretory rates of mutant chain from the cells. The osteoblasts will also provide an opportunity to examine non-collagenous mRNAs and proteins. "Genetic background" may affect OI phenotype in a variety of ways. Some of these effects may be regulatory and difficult to investigate. However, secondary alterations in the amounts of interacting matrix proteins are reasonable and accessible candidates.

9) For the cases of Ehlers-Danlos Syndrome with evidence of structural abnormality in type I collagen but without clinical evidence of bone disorder, our goal is to identify the associated mutations at the molecular level.

For the four cases whose electrophoretic abnormality persists in unmodified chains, we consider as prime candidates amino acid substitutions in the X or Y positions of the sequence involving cysteine, proline or lysine residues. Standard protein techniques and analysis of PCR products by hybrid analysis or denaturing gels will be utilized to localize the mutations prior to sequencing.

We will continue our collaboration with Dr. Yeowell at Duke University to investigate the molecular defect(s) of lysyl hydroxylase in the fibroblasts of our patient with type VI EDS.

10) We will continue to investigate the cases of Ehlers-Danlos IV with reduced amounts of type III collagen. If the mutant type III collagen is incorporated into triple helix, we may be able to further localize the abnormality by CNBr peptides. Type III collagen may be separated from the much larger amounts of type I collagen produced by these cells by selective salt precipitation. We will then pursue the abnormality by PCR amplification of the appropriate region of type III collagen mRNA. We have prepared amplification primers along the chain and have also subcloned overlapping segments of type III collagen cDNA into pGEM vectors for the production of riboprobe for hybrid analysis. If the mutant type III collagen protein is not incorporated into the triple helix, it will be necessary to investigate the entire type III cDNA by PCR amplification and hybrid analysis.

11) We will be continuing our clinical protocol consisting of a treatment trial of growth hormone in OI. We hope to enroll at 5 new patients by October 31, 1995 bringing the number of treated patients to 25 and closing out the study. In this year, the previously enrolled patients will complete the high dose phase of treatment. We will then be able to compare preliminary endocrine evaluations, and pre- and post-treatment bone density, bone morphometric properties, matrix peptides and growth factors in the group of OI children whose bone had a positive response to hormonal stimulation and in the non-responder group. Our goals are (a) to be able to predict which OI children will benefit from growth stimulation, and (2) to determine whether growth hormone exposure has a beneficial effect on OI bone density or bone morphometric properties apart from a stimulatory effect on linear growth.

12) We are planning to expand our investigations on growth in OI by submitting protocols dealing with the plateau phase of OI growth (age 1-4 yrs) and puberty. We have made the observation that growth of types III and IV OI children plateaus between ages 1-4 years. For the type IV children, it then resumes, often at a normal rate. To our knowledge, this time-limited phase of minimal growth has been described

only in OI. We are planning to submit a protocol for sequential endocrine evaluation of this age range. The clinical importance of this study is that elimination of the plateau phase would eliminate extreme short stature in type IV OI.

For the pubertal OI type III and IV children, we plan to study the effect of pubertal delay (via Lupron suppression) on final adult stature, bone density and spine curvature and compression. The clinical benefits include the possibility of increased final adult stature and increased thoracic height. Increased thoracic height may delay or eliminate the adult respiratory complications of OI.

13) We plan to submit a clinical protocol to enable us to document the effects of an aggressive rehabilitation approach to OI on the secondary features of OI. Children age 5-15 years will be followed at regular intervals for neurologic features (enlarged ventricles and basilar invagination), status of spine (scoliosis and compressions), pulmonary function, and cardiac function. This protocol will provide crucial natural history data and also serve to keep intact our study population for other investigations.

14) We are continuing our collaborative interest in the physical rehabilitation of children with OI in collaboration with Dr. Gerber, Chief, PM & R Department, NIH CC. The data analysis from the pairwise-crossover protocol in which OI children, who had attained ambulation using braces, were weaned from their braces will be completed. We are now recruiting patients for a prospective randomized trial of bracing in OI children under age 3 years who have not yet attained ambulation.

15) We will continue our collaborative study for the development of alternative rodding materials. In the area of resorbable rods, we will use the rodding material which has been formulated by Richards and Co. to stabilize femur fractures in rabbits. Resorbable rods will allow OI bone to gradually resume full weight bearing and avoid a surgery for removal.

Animal Protocols:

91-047	Marini	New Materials for Intramedullary Rods

Clinical protocols:

92-CH-34	Marini	Treatment Trial of Growth Hormone in Osteogenesis Imperfecta
89-CC-116	Gerber	Study of the Effects of Bracing on Ambulation, Bone, Density and Functional Activity in Children with OI

Publications:

Byers PH, Marini JC, GerberL. Frontiers in rehabilitation medicine: Osteogenesis Imperfecta, overview of a conference. Conn Tissue Res; in press.

Charnas LR, Marini JC. Neurologic profile in osteogenesis imperfecta. Conn Tissue Res; in press.

Marini JC, Bordenick S, Chrousos GP. Endocrine aspects of growth deficiency in OI. Conn Tissue Res; in press.

Principal Investigator: Joan C. Marini, M.D., Ph.D.
Heriatable Disorders Branch, NICHD

Title:

92-CH-0034 - "Growth Hormone Therapy in Osteogenesis Imperfecta"

Hypothesis: Growth deficiency is a cardinal feature of severe osteogenesis imperfecta (OI) and a frequent feature of mild to moderate forms of this disease. We have investigated the status of hormones related to growth in 28 short children, 14 males and 14 females, with various types of OI. Thirteen children had Sillence type III OI, 12 had type IV OI and 3 had type I. Evaluation included 24 hour q 20 minute sampling for measurement of unstimulated growth hormone secretion, growth hormone releasing hormone stimulation, somatomedin generation test, Argenine-Insulin tolerance test, and L-Dopa stimulation test. Abnormalities in the endocrine test patterns were found in a subgroup of the children studied. A group of children with decreased responsiveness to GRH also had significantly lower 24 hour time-integrated GH concentrations than children with normal GRH responsiveness. Overall, however, mean 24 hour GH vaalues and mean peak amplitude for OI children were within the normal range. In the Somatomedin generation test, the OI children as a group showed a blunted resposne, with 18/28 having less than a 2 fold stimulation of somatomedin-C by GH, but without correlation to other endocrine tests.

We conclude that abnormalities of the growth hormone - somatomedin axis exist in a subgroup of children with OI and some OI children might benefit from treatment with growth hormone.

STUDY DESIGN: We proposed to treat 40 children with types I, III, or IV OI with synthetic growth hormone (Somatropin) to determine (1) the range and duration of growth responsiveness of OI bone to growth hormone; (2) the correlation of this growth responsiveness with the results of endocrine evaluation of the growth hormone - somatomedin axis; and (3) the effect of growth hormone on the density and morphometric parameters of OI bone. Participants will complete an endocrine evaluation consisting of a Clonidine stimulation test, a 24 hour evaluation of GH secretion, and a somatomedin generation test. Bone age and structural status of the spine and long bones will be assessed radiographically. Clinical growth will be determined with 10 sequential morning stadiometer heights. All children will be treated with Somatropin for 12 months; those children who have not experienced a growth response after 6 months will be treated with a double dose of Somatropin for the remaining 6 months of therapy. All children who have experienced a positive growth response to Somatropin, defined as a 50% or greater increase in growth rate, during the first year of the protocol will be treated for an additional year to determine the duration of growth responsiveness.

CURRENT RESULTS: Of the 20 children who have received treatment in this protocol, all have completed 12 months of treatment. All but 1 child were increased to high dose during the 2nd 6 month phase. A total of 11 children experienced a 50% or greater increase in linear growth rate, and are continuing to respond positively during the 2nd 12 months.

For all children treated, ther is a highly significant rise in IGF-1 and IGFBP-3 values from baseline to 6 months or 12 months. There was no difference in the response of IGF-1 or IGFBP3 between children who increased their growth rate and those who did not increase their growth rate. Preliminary evaluation of IGFBP2 values at 6 months of treatment shows an increased level in responders and a decreased level in the non-responder group; the difference between the two groups is statistically significant. There is also a significant increase in alkaline phosphatase from baseline to 12 months for all treated patients, but this does not differentiate those children with positive linear growth response.

There are at least transient increases in the levels of osteoblast synthetic product osteocalcin, suggesting successful stimulation of the osteoblasts. At a year of treatment, the L2-L4 vertebral bone density is increased for growth responders as compared to non-responders, and the bone morphometric studies show increased bone volume and mineralized bone volume and mineralized bone volume per tissue volume.

CURRENT CONCLUSIONS: We have shown that some children with types I, III, and IV OI will respond to growth hormone treatment with increased linear growth rate; increased hormone levels, and positive changes in bone morphometrics but our preliminary analysis has not given us distinct criteria for which children will respond.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00415-05 HDB

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Physiology and Molecular Biology of Arachidonate Metabolism and CFTR Mutation in CF

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Anil B. Mukherjee, Ph.D., M.D.	Head	HDB, NICHD
Others:	Peter Yuan, Ph.D.	Visiting Fellow	HDB, NICHD
	Gopal C. Kundu, Ph.D.	IRTA Fellow	HDB, NICHD

COOPERATING UNITS *(if any)*
University of Pittsburg
Bureau of Biologics, FDA
University of California, San Diego

LAB/BRANCH
Heritable Disorders Branch

SECTION
Section on Developmental Genetics

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been merged with the main project #Z01 HD 00910-16 HDB

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00417-05 HDB

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Basis of α1-Antitrypsin Deficiency

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mark Brantly, MD.	Senior Staff Fellow	HDB, NICHD
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	N. Novoradovskaya, Ph.D.	Visiting Fellow	HDB, NICHD
	V. De Gouvea, Ph.D.	Special Volunteer	HDB, NICHD
	S. Ong, MS.	Biologist (Ebon)	HDB, NICHD
	D. Hoang	Special Volunteer	HDB, NICHD

COOPERATING UNITS *(if any)*

See attached

LAB/BRANCH
Heritable Disorders Branch

SECTION
Unit on Genetic Disorders of Secreted Proteins

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS: 3.25	PROFESSIONAL: 2.25	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(TERMINATE)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00910-16 HDB

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Uteroglobin, Phospholipase A_2, and Osteopontin Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Anil B. Mukherjee, M.D., Ph.D.	Head	HDB, NICHD
	Gopal C. Kundu, Ph.D.	IRTA Fellow	HDB, NICHD
	Gary Zhang, M.D., Ph.D.	IRTA Fellow	HDB, NICHD
	Peter Yuan, Ph.D.	Visiting Fellow	HDB, NICHD
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	Ijeoma Nwaeze	Stay-in-School	HDB, NICHD

COOPERATING UNITS *(if any)*
Georgetown University, McGill University, Harvard Medical School and Beth Isreal Hospital, Naval Research Laboratory, Washington Hospital Center/Medlantic Research Foundation, University of Florence, Italy.

LAB/BRANCH
Heritable Disorders Branch

SECTION
Developmental Genetics

INSTITUTE AND LOCATION
NICHD, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS: 4.5	PROFESSIONAL: 4.0	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have:(a) successfully disrupted uteroglobin (UG) gene in mice by homologous recombination and obtained germline transmission in F1 progeny; (b) developed an animal model expressing UG-antisense mRNA at high copy number; (c) cloned and characetrized the entire human UG gene; (d) delineated that UG gene expression in the rabbit uterus and in fetal lung occurs throughout gestation and that in the uterus this gene expression dramatically declines just prior to parturition, suggesting an important physiological role for this protein; (e) established and characterized two temperature-sensitive epithelial cell lines derived from the rabbit trachea and alveoli which differentiate *in vitro* and express UG and osteopontin genes;(f) resolved the crystallographic structure of recombinant hUG at 2.1 Å resolution; (g) resolved the solution structure of recombinant hUG by multidimensional NMR and CD; the results suggest that this protein is nearly identical to rabbit UG; (h) discovered that hUG inhibits cellular invasion via a novel high-affinity cell surface binding protein on NIH 3T3 and human trophoblast cells and human choriocarcinoma cells appear to lack this receptor and consequently, their invasiveness is not reduced by hUG; (i) demonstrated that hUG transcriptionally downregulate cyclooxygenase I & II via its receptor; (j) developed a mouse model which expresses osteopontin (OPN) antisense mRNA at high level; (k) determined that group I PLA_2-induced arachidonic acid release from NIH 3T3 cells may be receptor mediated; (l) demonstrated that group I PLA_2 can induce cellular invasion via its high-affinity receptor; (m) using both *in vitro* as well as *in vivo* assays demonstrated that cells lacking proto-oncogene *c-src* fail to express OPN gene; since OPN is required for normal osteoclast attachment to the osteoid, a process essential for bone remodeling, our results, at least in part, explains why *c-src*$^{-}/_{-}$ mice would develop osteopetrosis and (n) delineated that there is an intrinsic abnormality in epithelial cells derived from cystic fibrosis patients carrying ΔF508 mutation in cystic fibrosis gene, raising the possibility that novel strategies to control inflammation in the airway of these patients may include inhibition of PLA_2 activity.

Project Description:

I. Objectives:

Diseases caused by over-activation or suppression of immune responses are important causes of morbidity and mortality. Although the familial nature of several primary inflammatory diseases (eg. atopic asthma, rheumatoid and juvenile rheumatoid arthritis, autoimmune uveitis and psoriasis) is well recognized, the molecular and genetic mechanisms of pathogenesis of these diseases are still not clearly understood. Investigations in the general area of cell biology, immunology, molecular biology and genetics of inflammation have led to the discovery of genes which appear to play important role(s) as biological modulators (i.e. activators and inhibitors) of inflammation. For several years, one of the objectives of our laboratory has been to investigate the structure-function relationships of several genes and their products which directly or indirectly modulate immune and inflammatory processes. These include genes encoding phospholipases A_2 (PLA_2; E.C.3.1.1.4), the uteroglobin (UG)/Clara cell 10kDa (cc10kDa) family of proteins and osteopontin (OP). Within the last year we discovered a high-affinity cell surface receptor for human uteroglobin. Thus, our overall objectives also include: (a) cloning and characterization of the cDNA coding for this receptor and (b) delineation of the mechanism(s) of signal transduction via this receptor pathway in health and in disease.

A thorough knowledge of the mechanism(s) of action and regulation of these genes and their products is expected to improve our understanding of the molecular and genetic basis of some of the above mentioned diseases that affect a significant portion of our population and facilitate the development of rational and novel therapeutic approaches. To achieve these objectives, we have focused on investigations, the methods of approach and results of which are summarized below:

METHODS EMPLOYED:

State of the art methodologies of recombinant DNA technology, protein chemistry, cell biology and immunology are used. More specifically, we use all techniques related to gene cloning and sequencing, PCR, RT-PCR, *in situ* hybridization, Northern, Southern and Western blotting, generation of transgenic mouse model and gene targeting by homologous recombination ("knock out"), expression of recombinant proteins in *E. coli,* site-directed mutagenesis, radioimmunoassay, ELISA, tissue culture, cell invasion assay, immunohistochemistry and immunofluorescence. In collaborative studies, we applied X-ray crystallography and multidimensional Nuclear Magnetic Resonance (NMR) for structure-function analysis of recombinant proteins. Advanced enzymological and kinetic analyses are also used.

PROGRESS REPORT:

During the period since our last annual report we have accomplished the following:

a. **Targeted disruption of the uteroglobin (cc10kDa) gene in mice by homologous recombination:** While many biological effects of uteroglobin have been described, the physiological role(s) of this protein remains obscure. In order to determine the physiological role of this protein, we undertook the targeted disruption of UG gene in mice by homologous recombination. The mouse gene, like its human counterpart, consists of three exons and one intron. By using standard techniques, we have successfully disrupted this gene in ES cells and isolated and characterized three different ES clones. When these cells were injected in the

blastocyst and transferred them to surrogate mothers live chimeric progeny were born. The founders (chimeras) were further mated with C57/BL6j and obtained F1 mice, a number of which tested positive (heterozygote) for carrying the null mutation in UG gene (i.e. $UG^{+}/_{-}$). The F1 mice carrying the mutation attested that successful germline transmission of the mutation has been achieved. Presently, we are mating these heterozygotes (F1 X F1) obtained from each of the three types of founders to generate mice homozygous for UG gene disruption (i.e. $UG^{-}/_{-}$). These mice will be used for phenotype analyses.

b. **Development of transgenic mice expressing uteroglobin (cc10kDa) antisense-mRNA to determine the physiological role(s) of this gene:** Many important biological effects of uteroglobin reported so far, suggest that one outcome of homologous disruption of this gene may be fetal death. In the event this occurs, we have designed and generated transgenic mice carrying UG cDNA in an antisense orientation under the regulation of MMTV-LTR promoter. Since MMTV-LTR is a dexamethasoone-inducible promoter the level of the antisense mRNA is expected to be very high when the animals are treated with dexamethasone. Thus, the antisense transgenic mice are expected to develop and live normally until such time when they are treated with dexamethasone. We have obtained 3 founders (2 females and 1 male). These mice have been bred with C57BL/6j mice in order to establish 3 independent lines of progeny. This is done because the antisense transgene may be integrated in different chromosomes or different loci in the same chromosome and if all three lines express the same phenotype it would be concluded that it is due to the expression of the antisense mRNA and not as a result of different sites of integration of the transgene in different founders. We have generated a population of these mice in which high copy number of the transgene integrated to the genomic DNA could be detected by Southern hybridization. These mice are now being tested for the expression of the phenotype after dexamethasone induction of the antisense UG mRNA.

c. **Cloned and characetrized entire human UG gene:** A human genomic library constructed with placental DNA cloned into the *Xho I* site of the FIX II was obtained from Strategene. Approximately 1,000,000 clones were plated at high density and screened with the 367 bp hUG probe. Three primary positive clones were detected and picked. The cones were given the desinations: AM4-1, AM4-2 and AM4-3. These three clones obtained from primary screening were subjected to secondary and tertiary screening and Clones AM4-1 and AM4-2 were chosen for further characterization. Lambda DNA containing the hUG gene froom these two clones were purified and was digested with *Bam H I, Hind III, EcoR I* and *Not I.* Clones AM4-1 and AM4-2 exhibited different restriction patterns with *BamH I, Hind III, EcoR I* and*Pst I* digests. The *Not I* digest cuts out the insert intact, leaving a small portion of the vector on either end. The sizes of inserts in AM4-1 and AM4-2 are approximately 19kb and 16.5kb, respectively. Purified clone DNA from AM4-1 and AM4-2 was digested to completion with *Not I*, followed by complete and partial digests with *BamH I, EcoR I, Sac I* and *Xba I.* Digests were electrophoresed in 0.7% agarose gels, Southern transferred and hybridized with T7, T3, hUG 5'flanking region-derived nucleotides and 367bp *Pst I*fragment probes, individually. Restriction maps were constructed for both clones based upon the fragments hybridized with T7 and T3 probes. Specific restriction fragments of clone AM4-1 were identified which hybridized with hUG 5' flanking region and 367bp *Pst I* fragment probes. Neither probe hybridized with clone AM4-2 restriction fragments. This was confirmed by dot blot analysis where hUG 5' flanking region and 367bp *Pst I* fragment probes bound to AM4-I DNA but not AM4-2 DNA. A 4.2kb DNA fragment from AM4-1 containing the entire hUG gene was sequenced. We found that

similar to rabbit UG, human UG gene consits of three exons and two introns spanning 4.2 kb of DNA. Intron/exon structures of the hUG are generally very similar to rabbit UG gene.

d. **Uteroglobin gene expression continues throughout gestation in the rabbit uterus and abruptly stops just prior to parturition while in the fetal lung UG expression increases with increasing gestational age:** We and others have previously reported that UG expression in several tissues including the endometrium has an inverse relationship to eicosanoid levels. This is because of the potent inhibitory effects of UG on phospholipase A_2(PLA_2) enzyme activity, essential for eicosanoid production. It has been reported that UG gene expression in the uterus reaches a peak on the fifth day of gestation and by the tenth day there is no detectable UG in the endometrium of the uterus. Contrary to this report, we found that rabbit endometrial expression of UG mRNA as well as protein occurs throughout gestation and abruptly stops a day prior to parturition. In addition, we found that UG gene expression is first detectable in the fetus on day 17 of gestation and the level continues to rise until a very high level is reached at parturition. These findings indicate that UG gene expression may have a physiological role in regulating the onset of labor and maturity of the fetal lung. We previously reported the expression of UG gene in the surfactant-producing alveolar type II cells and suggested that UG may protect surfactant phospholipids from degradation by a lung-specific PLA_2. Our present results appear to support and strengthen this concept.

e. **Transient expression of uteroglobin but sustained expression of osteopontin gene in SV40 ts mutant-induced immortalized epithelial cells from the rabbit trachea:** Uteroglobin gene expression in the respiratory tract of all mammals, studied so far, is constitutive. During the past several years we have immortalized epithelial cell lines, from several organs which are known to express UG, by transformation with temperature-sensitive SV40 virus (A_{255}). In the earlier passages these cells expressed UG at a level similar to the same cells *in vivo*. But after 10-12 passages we noticed a decline in UG mRNA as well as protein levels in these cells. Since UG and osteopontin (OPN) are produced by respiratory epithelia and since OPN has been reported to be associated with transformed and malignant phenotype, we sought to determine whether there is a relationship between the expression of UG and OPN genes in young rabbit tracheal (YRT) epithelia and the same cells transformed and immortalized by transformation (YRT-1) by SV40 ts mutant transfection. We found that normal tracheal epithelial cells express UG gene at a high level while OPN gene expression is barely detectable. After several passages after transformation this pattern reverses itself: the YRT-1 cells express higher levels of OPN mRNA and protein but virtually no UG gene product is detectable. In addition, when the cells were stimulated with phorbol myristate acetate (PMA), a tumor promoter, very high level of OPN mRNA but not UG mRNA could be detected. These results indicate that transformation of the tracheal epithelial cells down regulates UG but up regulates OPN gene expression. Ongoing UG gene transfection experiments using YRT-1 cells may delineate the exact role UG and OPN genes play in maintaining the transformed phenotype of these cells.

f. **Resolution of recombinant human uterogobin structure by X-ray crystallography:** In light of the fact that human uteroglobin has recently been found to have a high-affinity binding on several cell types and that binding of hUG onto this receptor affects cellular invasion of extracellular matrix prompted us to determine the structure of this protein. Delineation of its structure will allow us to determine the region of hUG molecule involved in receptor-ligand interaction, knowledge of which is would facilitate our understanding of the signal transduction

via this hUG receptor. This knowledge may facilitate development of hUG receptor agonists which may be clinically useful in preventing cellular migration and invasion in inflammatory as well as in neoplastic diseases. In collaboration with Drs. Ward and Pattabiraman, we have resolved the human uteroglobin structure at 2.1Å resolution of X-ray crystallography. The monoclinic crystals used for diffraction studies yielded lattice constants a=69.2Å, b=83.0Å, c=58.3Å and β=99.7°. These results suggest that the secondary structures of rabbit and human UG are extremely similar, if not identical. Attempts are now being made to obtain even finer resolution of the crystal structure of hUG.

g. **Resolution of the solution structure of recombinant human uteroglobin by multidimensional NMR**: Since hUG is a soluble protein, it is important to determine the conformation of this protein in solution in order to understand what regions (or domains) of this protein are exposed to the solvent and therefore, available for interaction with other molecules. We expressed both unlabeled as well as ^{15}N and $^{15}N/^{13}C$-labeled recombinant hUG in *E.coli.* These proteins were purified to homogeneity and NMR studies were performed in collaboration with Dr. Gaetano Barbato at the IRBM, Rome Italy. Preliminary results of both circular dichroism (CD) spectroscopy as well as NMR suggest that hUG is a highly helical protein in solution. The hUG molecule in solution was found to be extremely stable. The apparent T_m appears to be above 70°C and a complete recovery of the native folding was possible at pH 6.2. The results of CD spectroscopy and NMR are in good agreement as regards to its T_m and α-helical structure. A comparison of these data with those from rabbit UG indicates that these two molecules are nearly identical structurally. Since our preliminary results of site-directed mutagenesis studies indicae that mutation of one lysine residue in α-helix 3 abolishes PLA_2 inhibitory activity of hUG, studies in progress are attempting to determine what structural constraints are brought about as a result of such mutations.

h. **Human UG inhibits cellular invasion via its high affinity cell surface binding site:** The mechanism(s) that regulates invasion of trophoblasts through the uterine epithelium during embryo implantation and nidation in hemochorial placental mammals is poorly understood. While limited trophoblast invasion is essential for the establishment of normal pregnancy, dysregulation of this process may contribute to the pathogenesis of choriocarcinoma, a highly invasive and lethal form of cancer arising from the trophoblasts. We have previously demonstrated that rabbit uteroglobin (UG), a cytokine-like, antiinflammatory protein, produced by the endometrial epithelium during pregnancy, has a potent antichemotactic effect on neutrophils and monocytes *in vitro*. Here, we report that recombinant human UG (hUG) dramatically suppresses invasion of human trophoblasts and NIH 3T3 cells through an artificial basement membrane (Matrigel) *in vitro*, but has no effect on that of human choriocarcinoma cells. We identified, for the first time, a novel, high-affinity, high molecular weight (~190kDa), non-glycosylated hUG-binding protein, readily detectable on human trophoblasts and NIH 3T3 cells but totally lacking on choriocarcinoma cells. Taken together, these results raise the possibility that (i) hUG plays a critical role in regulating cellular invasiveness, at least in part, via its novel cell surface binding site, and (ii) some of the numerous biological activities of proteins of the UG family, reported so far, may be mediated via this novel binding site.

i. **Human UG transcriptionally down-regulates cuclooxygenase I and II enzymes:** We have previously demonstrated that human uteroglobin inhibits both group I and II phospholipase A_2 (PLA_2) activities. However, recent data from other laboratories suggest that group I PLA_2

(PLA_2-I) also stimulate prostaglandin E_2 (PGE_2) production in several cell types in which a novel receptor for PLA_2-I has been discovered. Thus, we sought to determine whether hUG (an antiinflammatory protein) has any effect on cyclooxygenase enzymes (COX-I & II) which are responsible for converting arachidonic acid (AA) generated by PLA_2 catalysis of cell membrane phospholipids into proinflammatory lipid mediators (eg. PGE_2). We found that hUG treatment of MC3T3 cells not only lowers the PGE_2 level but also the COX-I and II mRNAs are also down regulated. These results suggest that hUG may transcriptionally regulate COX-I and II enzymes and lowers PGE_2 levels. A two-hybrid system is currently being used to delineate the mechanism of transcriptional down regulation of these two important enzymes in the inflammatory pathway.

j. **Developed a antisense transgenic OPN mouse model:** Last year we described that a mouse model is being generated in which the OPN cDNA in antisense orientation under the regulation of MMTV-LTR promoter was the transgene. In collaboration with Dr. Barid Mukherjee, McGill University, Canada, we have now established a colony of these mice. Preliminary characterization of these mice indicate that these mice develop severe abnormality in mammary gland differentiation. Since MMTV-LTR is known to target transgenes to the mammary gland and since OPN is expressed at a high level in this gland and detectable in milk, these preliminary results suggest that OPN gene expression is important for mammary gland differentiation. Ongoing studies will also focus on any abnormalities in bone and related organs for abnormalities and delineate the possible mechanism of this important phenotype.

k. **Determined that PLA_2-I-mediated arachidonic acid release by NIH 3T3 cells involves a receptor-mediated mechanism:** Group I pancreatic phospholipase A_2 (PLA_2 I) is primarily a digestive enzyme. Recently however, in addition to its catalytic activity a receptor-mediated function has been described for this enzyme. PLA_2 I binding to its receptor induces cellular chemokinesis, proliferation and smooth muscle contraction. This enzyme also induces the production of prostaglandin E_2 in certain cells and may have a proinflammatory role. However, despite its ability to hydrolyze phospholipids in *in vitro* assays, PLA_2-I does not efficiently catalyze release of AA from intact cells. Here, we demonstrate that while short-term exposure of NIH 3T3 cells to PLA_2-I is ineffective, exposure of 6 h or longer significantly increases the basal release of AA. Dose-response curve of PLA_2-I-induced AA release was saturable with an EC_{50} of 14.01 ± 1.36 nM (n = 3). [^{3}H]-AA was preferentially released over [^{3}H]-oleic acid by PLA_2-I. PLA_2-I, inactivated with 4-bromophenacyl bromide, was fully capable of mediating AA release. These data suggest that a non-catalytic, receptor-mediated mechanism is involved in PLA_2-I-induced AA release in NIH-3T3 cells. This release of AA is not dependent on protein kinase C or Ca^{2+} concentration. Comparison of the effect of PLA_2-I with those of ATP and platelet-derived growth factor indicates that each of these agonists regulates AA release via independent pathways. Neither the basal enzymatic activity of the 85-kDa cytosolic PLA_2 nor the protein level of this enzyme was affected by treatment of cells with PLA_2-I. However, the increase in basal enzymatic activity of 85 kDa PLA_2 due to protein kinase C activation was further enhanced by pretreatment of cells with PLA_2-I. We conclude that: (a) short-term exposure of cells to PLA_2 I does not cause measurable AA release; (b) release of AA from intact cells by this enzyme requires long-term exposure; (c) AA release is not mediated by a direct catalytic effect of PLA_2 I and (d) AA release by PLA_2 I is accomplished via a receptor-mediated process. Taken together, these results raise the possibility that PLA_2 I, in addition to

its digestive function, may also contribute to aggravate preexisting inflammatory processes and/or to initiate new ones when chronic exposure of cells to this enzyme occurs.

l. **Invasion of the extracellular matrix by NIH 3T3 cells via PLA$_2$-I receptor stimulation:** Phospholipases A$_2$ (PLA$_2$s) play key role(s) in the production of lipid mediators of inflammation. Structurally, extracellular low molecular weight (~14 kDa) PLA$_2$s belong either to group I or group II. Besides its catalytic function, group I PLA$_2$s (PLA$_2$-I), via its receptor, induce cell proliferation, chemokinesis and airway and vascular smooth muscle contraction. Here, we report that porcine pancreatic PLA$_2$ induces dramatic invasion of artificial extracellular matrix (Matrigel^{TM}) *in vitro* in a dose-dependent manner. Catalytic activity of PLA$_2$ does not appear to be essential for inducing this invasive effect. Moreover, a close similarity between the ED$_{50}$ for porcine pancreatic PLA$_2$-induced invasion and the Kd values for PLA$_2$-I binding to its receptor further indicates that this effect is receptor-mediated. Binding as well as affinity-crosslinking experiments confirmed that NIH 3T3 cells bind *Naja naja* PLA$_2$, mannose-BSA, galactose-BSA and NAG-BSA with high affinity and specificity as these ligands displace ^{125}I-PLA$_2$-I from its binding site. Western blot analysis revealed immunological identity of this receptor with fibronectin type II-like domain, common to both rabbit and bovine PLA$_2$ receptors. A 4.2 kb PLA$_2$-I receptor-mRNA in NIH 3T3, Swiss 3T3 and mouse osteoblastic (MC 3T3) cell lines was detected by Northern hybridization. Since cellular invasion of ECM plays critical roles in embryonic implantation, inflammation and cancer cell metastasis, this novel effect may suggest important role for extracellular PLA$_2$s and their receptor-mediated function(s) in physiological as well as pathological processes.

m. **Osteopetrosis in protooncogene c-src disrupted mice may be due a lack of osteopontin gene expression:** Disruption of proto-oncogene c-*Src* in mice (i.e. c-*Src*$^{-}/_{-}$), by homologous recombination causes osteopetrosis due to impaired bone resorption by osteoclasts. Osteopontin (OPN), a secreted, non-collagenous, glycosylated phosphoprotein, is essential for osteoclast adherence to bone matrix, a prerequisite for bone resorption. We found that cells derived from c-*Src*$^{-}/_{-}$ osteopetrotic mice do not synthesize OPN, but its expression is inducible by epidermal growth factor (EGF) or 12-O-tetradecanoyl phorbol-13-acetate (TPA) treatment. Using c-*Src*$^{-}/_{-}$, c-*Src*$^{+}/_{-}$ and a c-*Src* over-expressor cell line, CB, as well as tissue samples from c-*Src*$^{-}/_{-}$, c-*Src*$^{+}/_{-}$ and c-*Src*$^{+}/_{+}$ mice we have demonstrated that c-*src* plays a critical regulatory role in OPN gene expression in these cells, raising the possibility that lack of OPN, at least in part, may be responsible for the osteopetrotic phenotype in c-*Src*$^{-}/_{-}$ mice. Furthermore, our results indicate that contrary to published reports, EGF-mediated cell signaling may not require functional interaction between EGF-receptor and pp60$^{c\text{-}src}$.

n. **Inflammation in the airway of cystic fibrosis patients may be due to an intrinsic abnormality in PLA$_2$ gene expression:** Chronic inflammation in the airways is a suggested mechanism of destructive lung disease in cystic fibrosis (CF). Recently, it has been reported that long-term treatment of CF patients with high-dose ibuprofen significantly slowes the progression of destructive lung disease. Ibuprofen, at high doses, is a dual inhibitor of cyclo- and lipoxygenase enzymes which utilize arachidonic acid (AA) to generate potent lipid mediators of inflammation (eg. prostaglandins, leukotrienes etc.). However, it is not clear whether CF transmembrane conductance regulator (CFTR) mutation in CF epithelial cells is associated with abnormal AA release, although numerous reports suggest that eicosanoids and phospholipid metabolism may be altered in CF.We studied Ca^{2+}-induced release of arachidonic acid (AA),

by CF epithelial cell lines (CFPAC-1 and CFPAC-1:PLJ) carrying ΔF_{508} mutation in CFTR gene. We compared these results with those from CFPAC-1:PLJ-CFTR, a cell line in which the ΔF_{508} mutation was corrected by wild type CFTR gene transfer and T84, a cell line which overexpresses wild type CFTR gene. We found that: (i) CF epithelial cells with ΔF_{508} mutation in CFTR show an intrinsic abnormality in Ca^{2+}-induced AA release as compared to controls, (ii) AA release appeares to be catalyzed by an as yet unidentified phospholipase A_2 (PLA_2), but not by the combined effects of phospholipase C and diacylglycerol lipase, and (iii) either correction of the CFTR-mutation by gene transfer or inhibition of PLA_2 activity by quinacrine correct the AA release abnormality. Our results suggest that one of the mechanisms of chronic inflammation in CF may involve an intrinsic abnormality in AA release by CF epithelial cells carrying ΔF_{508} mutation in CFTR. Moreover, these results raise the possibility that regulation of abnormal AA release by inhibiting PLA_2 activity may potentiate the observed beneficial effects of ibuprofen.

COOPERATIVE RESEARCH AND DEVELOPMENT AGREEMENT (CRADA):

1. Knight, M. Collaborating Principal Investigator:Peptide Technologies Corp. Washington, D.C.

   Title of Project: Development of a radiometric assay for HIV-1 aspartic protease activity for screening potential inhibitors of this enzyme. Approved June 1, 1991.

Significance to Biomedical Research and to the Program Goals of the Institute:

Immunologically mediated inflammation and tissue injury characterize a number of relatively common human diseases such as bronchial asthma, rheumatoid and juvenile rheumatoid arthritis and autoimmune uveitis, to name a few. Together, these diseases affect a significantly large population of the US and the world. For instance, asthma is one of the most frequent causes of hospital admission for children and causes approximately 5,000 deaths per year in the US alone. In diseases like rheumatoid arthritis and asthma, inflammation has been suggested to play a primary pathogenic role. In other diseases, such as cystic fibrosis, inflammation in the airway aggravates the primary pathologic processes resulting from chloride-channel abnormality and causes significant increase in mortality and morbidity. Our discovery that the most commonly encountered CFTR gene mutation (ΔF508) in CF causes an intrinsic abnormality in arachidonic acid release may open new avenues of therapy for this devastating complication of cystic fibrosis. Although the primary inflammatory diseases [eg. rheumatoid and juvenile rheumatoid arthritis and atopic (allergic) asthma] have a familial basis, the exact genetic and molecular mechanisms underlying these diseases are not clearly understood.

Our most recent discoveries on the sub-chromosomal localization of the human uteroglobin gene in chromosome $11q^{13.1-13.2}$, suppression of chemoinvasion of trophoblasts but not choriocarcinoma cells by recombinant uteroglobin and inhibition of highly metastatic prostatic cancer cells by this protein may provide vital information on the physiological role of this protein. In addition, these results raise the possibility that UG may be a candidate disease gene possibly having a role in embryonic differentiation and cancer metastasis. Moreover, our recent iinvestigation on osteopontin gene is beginning to explain the etiology of some forms of osteopetrosis. The involvement of the three genes, studied in this Section (e. UG, OPN and PLA_2), in childhood and genetic diseases of profound Public Health implications make our research endeavor consonant with the mission of NICHD.

ANIMAL PROTOCOLS:

#92-037	Reagent Antibody Production
#93-007	Uteroglobin:(a) tissue-specific expression of the UG gene in developing rabbit and (b) possible therapeutic benefits of administration of artificial surfactant-recombinant human UG complex in the treatment of neonatal respiratory distress syndrome (RDS) in the rabbit.
#94-047	Determination of physiological role of uteroglobin by using UG-antisense transgenic mice.
#95-018	Determination of the physiological role of uteroglobin by gene targeting in mice.

LIST OF PUBLICATIONS:

1. Gerhardt, D.S., Kamarasova, T., Lawrence, E., Wu, J., **Mukherjee, A.B.** and Jones, C. Isolation of a large contig in chromosome $11q^{13}$: the possible location of Best's macular degeneration and Atopy genes. *Genomics (in press)*

2. Knight, M., Ito, Y., Chandrasekhar, B., Takahashi, K. and **Mukherjee, A.B.**: Purification of the human immunodeficiency virus-1 aspartyl protease substrate peptide.In: Amer. Chem. Soc. Symp. Series #593: *Modern Countercurrent Chromatography.* Conway, W.D. and Petroski, R.J. eds. Amer. Chem. Soc. Publ. **1995**. Chapter 10: pp111-118.

3. Mathews, J.H., Pattabiraman, N., Ward, K.B., Mantile, G., Miele, L. and **Mukherjee, A.B.** Crystallization and characterization of the recombinant human Clara cell 10kDa protein. *Proteins: Structure, Function and Genetics.*20:191-196, 1995.

4. Miele, L., Cordella-Miele, E., Mantile, G., Peri, A. and **Mukherjee, A.B**. Uteroglobin and uteroglobin-like proteins. The uteroglobin family of proteins. *J. Endocrinol. Invest.* 8:679-692, 1994.

5. Miele, L., Peri, A., Cordella-Miele, E. and **Mukherjee, A.B**. Non-radiometric ELISA-based quantitation and validation of polymerase chain reaction-amplified DNA, including detection of point mutations, without allele-specific amplification of ligation. *DNA Cell Biol.* 13(12):1233-1242, 1994.

6. **Mukherjee, A.B.**, Miele, L. and Pattabiraman, N.: Phospholipase A_2: Mechanism of catalysis and regulation. *Biochem. Pharmacol.* 48: 1-10, 1994.

7. **Mukherjee, A.B.**, and Miele, L. Design of immunomodulatory peptides based on active site structures. In: *Structure-based Drug Design (William Williams and David Weiner eds).* CRC Press, Inc. 1995, pp.237-261

8. Peri, A., Dubin, N., Dhanireddy, R. and **Mukherjee, A.B.** Uteroglobin gene expression in the rabbit uterus throughout gestation and in the fetal lung: Relationship between uteroglobin and eicosanoid levels in the developing fetal lung. *J. Clin. Invest. (In press).*

9. Su, L., Mukherjee, B.B. and **Mukherjee, A.B.** Expression of antisense osteopontin RNA inhibits tumor promoter-induced neoplastic transformation of mouse JB6 epidermal cells. *Oncogene* 10:2163-2169, 1995.

10. Ulane, M.M., Butler, J. DeB., Peri, A., Miele, L., Ulane, R. and Hubbard, V.S. Cystic fibrosis and phosphatidylcholine biosynthesis. *Clin. Chim. Acta, 230:109-116, 1994*

11. Xing, M., Miele, L. and **Mukherjee, A.B.** Arachidonic acid release from NIH 3T3 cells by group I phospholipase A_2: Involvement of a receptor-mediated mechanism. *J. Cell. Physiol. (In press).*

PROPOSED COURSE:

Our future research plans include the following:

a. To establish whether hUG gene mutation is associated with human diseases.

b. To fully characterize UG "knockout" mice for phenotypes.

c. To purify and characterize the human uteroglobin (hUG)-binding protein (putative receptor) and clone and characterize the cDNA and the gene coding for this protein;

d. To delineate the mechanism(s) of signal transduction via hUG receptor;

e. To further characterize the suppression of cellular invasion via hUG receptor-mediated pathway;

f. To develop strategies to inhibit abnormal arachidonic acid release in cells derived from patients with cystic fibrosis (CF) carrying ΔF508 mutation in CFTR gene;

g. To further characterize the relationship between c-Src and c-fos gene disruption, suppression of OP gene expression and development of osteopetrosis.

h. To delineate the role of osteopontin gene in mammary gland development in OPN-antisense transgenic.

i. Development of OPN-"knockout" mice to delineate the physiological role of OPN.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00912-16 HDB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Regulation and Cellular Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Janice Y. Chou, Ph.D.	Head	HDB, NICHD
Others:	Kei-Jian Lei, Ph.D.	Visiting Scientist	HDB, NICHD
	Margaret Chamberlin, Ph.D.	IRTA Fellow	HDB, NICHD
	Hungwen Chen, Ph.D.	Visiting Fellow	HDB, NICHD
	Tsuneyuki Ubagai, Ph.D.	Visiting Fellow	HDB, NICHD
	Chi-Jiunn Pan	Chemist	HDB, NICHD
	Brian C. Mansfield, Ph.D.	Adjunct Professor	HDB, NICHD
	Baochuan Lin	Adjunct Student	HDB, NICHD

COOPERATING UNITS *(if any)*

German Cancer Research Center (Drs. G Schutz); University of Florida (Dr. C-L Chen); Georgetown University (Dr. Y-W Chan); Duke University (Dr. Y-T Chen); University of North Dakota (Dr. R Nordlie)

LAB/BRANCH

Heritable Disorders Branch

SECTION

Cellular Differentiation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glycogen storage disease (GSD) type 1 is an autosomal recessive disease caused by the deficiency of glucose-6-phosphatase (G6Pase). Diagnosis of GSD type 1a currently is established by demonstrating the lack of G6Pase activity in the patient's biopsied liver specimen. Our cloning of the G6Pase gene and identification of mutations within the gene that cause GSD type 1a allow for the development of a DNA-based diagnostic method. We have characterized the G6Pase gene of 70 unrelated GSD type 1a patients and detected mutations in all except 17 alleles (88%). Sixteen disease-causing mutations were uncovered and the prevalent mutations in Caucasians, Hispanics, and Chinese were identified. This DNA-based diagnosis now permits prenatal diagnosis among at-risk patients and serves as a data-base in screening and counseling individual clinically suspected of having this disease.

We have characterized mutant G6Pases generated by near-saturation mutagenesis of codons 83, 222, and 295 known to be important for G6Pase catalysis. We showed that Arg-83 may be involved in stabilizing the enzyme-phosphate intermediate formed during G6Pase catalysis and Arg-295 may be involved in salt-bridge formation. Mutagenesis studies also show that His-119 is the phosphate acceptor in G6Pase catalysis.

A null G6Pase mutation in embryonic stem cells has been generated by targeted-disruption of the G6Pase gene. These genetically modified cells were used to establish G6Pase-deficient mutant mice for use as a model of GSD type 1a. Our studies show that the G6Pase-deficient mice mimic GSD type 1a in humans.

Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation and transsulfuration. α/β-MAT deficiency causes isolated persistent hypermethioninemia, unusual breath odor or neural demyelination. We have characterized the human α/β-MAT gene, identified several mutations in the gene of MAT deficient patients, and showed that these mutations partially inactivate MAT activity. These results establish the molecular basis of this disorder and allow for the development of DNA-based methods to diagnose hypermethioninemic individuals suspected of having abnormalities at this locus.

The pregnancy-specific glycoproteins (PSGs) form a large family of essential pregnancy proteins, but their biological function is unknown. We show that the PSG11s receptors are present on cells of the myeloid cell lineage, suggesting that one of function of PSG is to interact with cells of the maternal immune system.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The Objectives of this project are to understand the molecular genetics of glycogen storage disease (GSD) type 1 and methionine adenosyltransferase (MAT) deficiency, and the physiological functions of pregnancy-specific glycoprotein (PSG).

GSD type 1a is caused by the deficiency in glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis catalyzing the terminal step in gluconeogenesis and glycogenolysis. This laboratory has pioneered the molecular genetic studies of GSD type 1a and established the molecular basis of the type 1a disorder. Current areas of GSD research include: (1) to identify mutations in the G6Pase genes of GSD type 1a patients and to correlate the specific defects with clinical manifestations of the disease; (2) to clone the G6P translocase hypothesized to be deficient in GSD type 1b patients; (3) to delineate the structure-function relationship of G6Pase catalysis; (4) to generate a mouse model for studying the molecular mechanism(s) and pathophysiology of GSD type 1a and for developing effective therapies for this disease; and (5) to characterize the molecular mechanisms underlying tissue-specific and hormone-dependent expression of the G6Pase gene. The goals of this study are to understand the molecular mechanisms and pathophysiology of GSD type 1a, to develop novel therapeutic approaches for this genetic disease, and to understand the molecular mechanism(s) of G6Pase catalysis.

Methionine adenosyltransferase (MAT) deficiency is caused by the deficiency of α/β-MAT, a key enzyme in transmethylation, transsulfuration and the biosynthesis of polyamines. MAT deficiency is characterized by isolated persistent hypermethioninemia and, in some cases, unusual breath odor or neural demyelination. Current areas of MATD research include: (1) to identify mutations in the MAT gene of patients with isolated persistent hypermethioninemia with or without neural demyelination to determine if mutations in the α/β-MAT gene are associated with the phenotypes; and (2) to delineate the structure-function relationship of MAT catalysis. The goals of this study are to understand the molecular mechanisms of MATD and to investigate and diagnose hypermethioninemic individuals suspected of having abnormalities at this locus.

PSGs form a family of closely related proteins which are essential for pregnancy. Despite being present at high levels in the maternal blood, the roles of PSGs are unknown. The goals of this project is to identify and characterize the biological roles of the PSG and explore their clinical use, during pregnancy and for contraception.

Methods Employed:

The principal methods are: (1) all current methods in nucleic acid research, including mRNA and DNA isolation, cDNA library construction, Northern and Southern blot analyses, S1-mapping, ribonuclease protection analysis, polymerase chain reaction, *in situ* hybridization, nuclear run-off assays, CAT assays, gel retardation assays, footprinting assays, site-directed mutagenesis, DNA cloning, SSCP analysis, and DNA sequencing; (2) all current methods in tissue culture research; (3) protein isolation techniques, including gel filtration ion-exchange chromatography, and affinity chromatography; (4) enzyme assays, both spectrophotometric and radiometric methods; (5) radioimmunoassays; (6) establishment of differentiated mammalian cell lines by viral transformation; (7) immunoprecipitation, gel electrophoresis, and fluorography; (8) expression of cloned genes in the baculovirus system; and (9) gene targeting and the generation of knock-out mouse strains.

Major Finding:

A. Glycogen Storage Disease (GSD) Type 1

Glycogen storage disease (GSD) type 1a is caused by the deficiency of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis catalyzing the terminal step in gluconeogenesis and glycogenolysis. This autosomal recessive disorder is characterized by hypoglycemia, hepatomegaly, nephromegaly, growth retardation, delayed puberty, lactic acidemia, and hyperuricemia. GSD type 1 presents with heterogeneous symptoms and has been divided into five subtypes. These correspond to defects in the G6Pase catalytic unit (type 1a), the putative G6P translocase (type 1b), the putative phosphate/pyrophosphate translocase (type 1c), the putative glucose translocase (type 1d), and a putative G6Pase stabilizing protein (type 1aSP). Biochemically, the classification of type 1a and 1aSP patients is based on a low to undetectable G6Pase activity in liver biopsy samples and the classification of type 1b and 1c patients on a high and detectable activity in detergent-treated liver biopsy samples. Two models of G6Pase catalysis have been proposed to account for these observations. The conformation-substrate-transport model proposes that G6Pase is a membrane channel protein with both catalytic and substrate (or product) transport functions. On the other hand, the translocase-catalytic unit model suggests that G6Pase is a multi-component complex consisting of a catalytic unit, G6Pase, situated on the lumenal surface of the ER that gains access to substrates from the cytosol via associated translocases. To distinguish between the two models of G6Pase catalysis and to elucidate whether mutations in the G6Pase gene are responsible for other GSD subtypes, we characterized the G6Pase of type 1b, 1c, and 1aSP patients. We showed that the GSD type 1aSP patient is homozygous for the R83C mutation, the most prevalent mutation in type 1a patients, indicating that type 1aSP is a misclassification of GSD type 1a. In addition, we have shown that a deleterious mutation in the G6Pase gene is not associated with the GSD type 1b and 1c lesions, supporting the translocase-catalytic unit model of G6Pase catalysis. Our date indicate that there are at least two loci that control the rate of G6P hydrolysis *in vivo*.

We have previously identified six disease-causing mutations in the G6Pase gene of GSD type 1a patients. Of these, four mutations (R83C, R295C, G222R, and Q347X) are missense and one (Q347X) generates a truncated G6Pase of 346 residues. Human G6Pase is a hydrophobic protein of 357 amino acids. To further understand the roles and structural requirements of amino acids 83, 222, 295, and those at the carboxyl terminus in G6Pase catalysis, we characterized mutant G6Pases generated by near-saturation mutagenesis of the aforementioned amino acids. Substitution of Arg-83 with amino acids of diverse structures including Lys, a conservative change, yielded mutant G6Pase with no enzymatic activity. On the other hand, substitution of Arg-295 with Lys (R295K) retained high activity and R295N, R295S, and R295Q exhibited moderate activity. All other substitutions of Arg-295 had no G6Pase activity, suggesting that the role of Arg-295 is to stabilize the protein either by salt-bridge or hydrogen-bond formation. Substitution of Gly-222, however, remained functional unless a basic (Arg or Lys), acidic (Asp), or large polar (Gln) residue was introduced, consistent with the hydrophobic requirement of codon-222 which is predicted to be in the fourth membrane-spanning domain. It is possible that Arg-83 is involved in stabilizing the enzyme (His)-phosphate intermediate formed during G6Pase catalysis. There exist 9 conserved His residues in human G6Pase. His-9, His-119, His-252, and His-353 reside on the same side of the endoplasmic reticulum membrane as Arg-83. Whereas H119A mutant G6Pase had no enzymatic activity, H9A, H252A, and H353A mutant G6Pases retained significant activity. Substitution of His-119 with amino acids of diverse structures also yielded mutant G6Pase with no activity, suggesting His-119 is the phosphate acceptor in G6Pase catalysis.

G6Pase, located in the ER, contains the ER transmembrane protein retention signal, KK, at residues 354 and 355. The Q347X mutant, lacking the eleven carboxyl terminal amino acids, is enzymatically inactive. To examine the length of the carboxyl terminus required for G6Pase activity and the role of the KK signal, we examined phosphohydrolase activity of mutant G6Pases containing sequential deletions of carboxyl terminal residues. Deletion of amino acids 350 to 355 yielded mutant G6Pases retaining at least 40% of wild-type enzyme activity. However, a G6Pase of 348 residues (L349X) exhibited only 5% of wild-type G6Pase activity, demonstrating that the eight carboxyl-terminal residues beyond this codon, which contain an ER protein retention signal, are not necessary for its catalytic activity.

Diagnosis of GSD type 1a currently is established by demonstrating the lack of G6Pase activity in the patient's biopsied liver specimen. Recent cloning of the G6Pase gene and identification of mutations within the gene that cause GSD type 1a allow for the development of a DNA-based diagnostic method. Using single strand conformation polymorphism analysis (SSCP) and DNA sequencing, we characterized the G6Pase gene of 70 unrelated patients with enzymatically confirmed diagnosis of GSD type 1a and detected mutations in all except 17 alleles (88%). Sixteen mutations were uncovered which were shown by expression to abolish or greatly reduce G6Pase activity and are therefore responsible for the GSD type 1a disorder. R83C and Q347X are the most prevalent mutations found in Caucasians, 130X and R83C in Hispanics, and R83H in Chinese. The Q347X mutation has thus far been identified only in Caucasian patients, and the 130X mutation only in Hispanic patients. Our results demonstrate that the DNA-based analysis can accurately, rapidly, and non-invasively detect the majority of mutations in GSD type 1a. This DNA-based diagnosis now permits prenatal diagnosis among at-risk patients and serves as a data-base in screening and counseling patients clinically suspected of having this disease.

At present, treatments of GSD type 1a focus solely on controlling symptomatic hypoglycemia. Patients who follow careful metabolic control continue to have blood lactate, cholesterol, and triglyceride values above the normal range and kidney function eventually becomes compromised. It is not clear whether long-term complications including gout, hepatic adenomas, and osteoporosis will eventually develop in the patients despite good dietary intervention. To study the molecular mechanism(s) and pathophysiology of GSD type 1a and to develop an effective, alternative therapy for this disorder we constructed G6Pase-deficient mouse strains by homologous recombination in embryonic stem cells. Homozygous mutant mice mimic GSD type 1a in humans with clinical presentations of growth retardation, recurrent hypoglycemia, hepatomegaly, nephromegaly, lactic acidemia, hypertriglyceridemia, hyperuricemia and dysplasia of of the long bones in knee joint. Histologically, the G6Pase-deficient mice had marked glycogen storage in hepatocytes of the liver and proximal tubular epithelial cells of the kidney. Cytoplasmic lipid droplets were prominent in hepatocytes, another characteristics of GSD type 1a in humans. The majority of homozygous mutant mice died around 20 to 25 days of age. Necropsy analysis revealed extensive renal glomerular atrophy which were severe enough to contribute the death of the animals. The mouse line provides a suitable model for the development of somatic gene therapy for GSD type 1a.

B. Methionine Adenosyltransferase Deficiency

Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation, transsulfuration and the biosynthesis of polyamines. Three forms of MAT (α, ß, and γ) have been identified in mammalian tissues. The α and ß MATs, composed of homotetramers and homodimers, respectively, are expressed primarily in adult liver. Genetic deficiency of α/β-MAT causes isolated persistent hypermethioninemia and, in some cases, unusual breath odor or neural demyelination. However, the molecular mechanism(s) underlying this deficiency has not been clearly defined. To study the

molecular genetics of this disorder, we characterized the human α/β-MAT gene and showed that it is a single copy gene consisting of 9 exons and 8 introns. Using a combination of single-stranded conformation polymorphism (SSCP) and DNA sequence analyses, we characterized the α/β-MAT gene in three unrelated patients with enzymatically confirmed diagnosis of MAT deficiency. All three patients were found to contain mutations in the coding region of the α/β-MAT gene and each of these mutations partially inactivates MAT activity. These results establish the molecular basis of this disorder and allow for the development of DNA-based methodologies to investigate and diagnose hypermethioninemic individuals suspected of having abnormalities at this locus.

C. Pregnancy-Specific Glycoproteins

Pregnancy-specific glycoproteins (PSGs), which are the major placental proteins, and the carcinoembryonic antigens comprise a subfamily within the immunoglobulin superfamily. To understand the molecular mechanisms underlying the control of PSG expression, we characterized the promoter elements of a rodent PSG gene, rnCGM3, and showed that DNA elements at nucleotides -326 to -185 (PI) relative to the translation start site of rnCGM3 function as a promoter. The rnCGM3 PI promoter contains two placental factor binding sites, PISI and PISII. Both are transcription activation elements. To identify the protein factor binding to PISII, we screened a placental expression cDNA library with a rnCGM3-PISII probe (nucleotides -263 to -233) encompassing two overlapping palindromes (TGTTGCTCAACATGTTG) and demonstrated that the PISII-binding factor is C/EBPβ, a member of the leucine zipper family of transcription factors. Gel mobility shift and transient expression analyses showed that C/EBPβ and C/EBP isoforms, C/EBPα and C/EBPδ, bind to the PISII element and transactivate rnCGM3 gene expression. Deletion of PISII from the rnCGM3 PI promoter greatly reduced the basal as well as the C/EBP-activated rnCGM3 expression. Gel supershift assays demonstrated that C/EBPβ is the placental isoform that binds to the PISII site in rnCGM3. Moreover, C/EBPβ is expressed in high levels in the placenta, ovary, liver, lung, heart, and spleen, in contrast to C/EBPα which is expressed primarily in the liver and only low levels in the placenta. Our results demonstrate that C/EBPβ is one of the transcription factors that positively regulate rnCGM3 expression during pregnancy.

PSGs form a large family of essential pregnancy proteins, but their biological function is unknown. We have investigated whether one function of the PSG is to interact with cells of the maternal immune system. Normal human peripheral blood mononuclear cells, activated with phorbol ester, are shown to bind to purified placental PSG. This binding activity can be mimicked using a chemically synthesized peptide ligand containing the Arg-Gly-Asp (RGD) motif present in the N-terminal domain of PSG11s. The PSG11s receptors are present on cells of the myeloid cell lineage but not of the T-cell or B-cell lineages. The binding is mediated in part by the RGD motif and can be competed against by appropriate RGD-containing, but not RAD-containing ligands. Ligand binding requiring functional receptors with different binding characteristics have been demonstrated. The THP-1 receptor is identified by chemical cross-linking as a protein of 46 kDa, and affinity chromatography demonstrated the presence of three proteins of 32, 16.8, and 15.9 kDa, suggesting the receptor has multiple subunits.

Significance to Biomedical Research and the Program of the Institute

GSD type 1 is caused by the deficiency of G6Pase, the key enzyme in glucose homeostasis. This metabolic disease typically manifests during the first year of life with severe hypoglycemia, and hepatomegaly. Moreover, individuals with GSD type 1 exhibit growth retardation, delayed puberty, nephromegaly, lacticacidemia, hyperlipidemia, hyperuricemia, and in adults, a high incidence of renal failure and hepatic adenomas. Currently, four GSD type 1 subgroups exist which correspond to

defects in the G6Pase catalytic unit (1a), the glucose-6-P (1b), phosphate/pyrophosphate (1c), and glucose (1d) translocases. Characterization of the human G6Pase gene and the mutations causing G6Pase deficiency have established the molecular basis of GSD type 1a and provided vital information for developing novel therapies. The generation of animal model for GSD type 1a would be important for investigating the molecular mechanisms and pathophysiology of this disorder and for developing therapies. Characterization of the genes for the putative translocases and the mutations causing GSD type 1b and 1c are essential for understanding the molecular basis of these disorders and the mechanism of G6Pase catalysis.

Hepatic α/β-MAT deficiency is a genetic disorder with clinical symptoms of isolated persistent hypermethiominemia and an unusual breath odor in the adult patients. Most of these patients have been free of major clinical difficulties. Additional individuals with biochemical abnormalities suggestive of α/β-MAT deficiency have been identified and some patients developed abnormal neurological symptoms and neural demyelination that responded favorably to AdoMet therapy. Characterization of the MAT gene and mutations that cause MAT deficiency are essential for understanding the molecular basis of this disorder and the vital role of this enzyme in transmethylation and transsulfuration.

National statistics reveal that over 50% of pregnancies end in miscarriages or abortion. Therefore, understanding the role of pregnancy-associated protein factors in implantation, maintenance of pregnancy, and fetal development is fundamental to women's health. The characterization of the cis-acting elements in the 5'-flanking regions of PSG genes would allow us to identify the placenta-specific transcription factor(s) activated during pregnancy.

Proposed Course

1. Identification of mutations in the G6Pase gene of GSD type 1a patients and study of the structural-functional relationship of human G6Pase.

2. Delineation of the molecular mechanisms and pathophysiology of GSD type 1a using the G6Pase-deficient mice generated by homologous recombination in embryonic stem cells.

3. Development of gene therapy for GSD type 1a.

4. Characterization of the model for G6Pase catalysis.

5. Establishment of the molecular basis of the GSD type 1b disorder.

6. Establishment of an accurate, nonivasive, and gene-based prenatal diagnosis of GSD type 1a and potential carriers.

7. Identification of mutations in the α/β-MAT gene of deficient patients and study of the structural-functional relationship of human MAT.

8. Delineation of the functional roles of PSG during pregnancy.

Publications:

Chen H, Chen C-L, Chou JY. Characterization of two promoters of a rat pregnancy-specific glycoprotein gene. Biochemistry 1994;33:9615-9626.

Chen H, Lin B, Chen C-L, Johnson PF, Chou JY. Role of the Transcription Factor C/EBPβ in Expression of a Rat Pregnancy-specific Glycoprotein Gene. DNA Cell Biol, in press.

Hurst BS, Ziberstein M, Chou JY, Litman B, Stephens J, Leslie KK Functional estrogen receptors are present in human granulosa cells. J Clin Endocrinol Metab 1995;80:229-232.

Lei K-J, Chen Y-T, Chen H, Wong L-JC, Liu J-L, McConkie-Rosell A, Van Hove JLK, Ou HC-Y, Yeh NJ, Pan LY, Chou JY. Genetic basis of glycogen storage disease type 1a: prevalent mutations at the glucose-6-phosphatase locus. Am J Hum Gen, in press.

Lei K-J, Shelly LL, Lin B, Sidbury JB, Chen Y-T, Nordlie RC, Chou JY. Mutations in the glucose-6-phosphatase gene are associated with glycogen storage disease type 1a and 1aSP but not 1b and 1c. J Clin Invest 1995;95:234-240.

Lei K-J, Shelly LL, Pan C-J, Liu J-L, Chou JY. Structure-function analysis of human glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1a. J Biol Chem 1995;270:11882-11886.

Liu Z, Barrett EJ, Dalkin AC, Zwart AD, Chou JY. Effect of acute diabetes on rat hepatic glucose-6-phosphatase activity and its messenger RNA level. Biochem Biophys Res Commun 1995;205:680-686.

Rutherfurd KJ, Chou JY, Mansfield BC. A motif in PSG11s mediates binding to a receptor on the surface of the promonocyte cell line THP-1. Mol Endocrinol, in press.

Sirvastava RK, Zilberstein M, Ou JS, Mayo KE, Chou JY, Gibori G. Development and characterization of a SV-40 transformed temperature sensitive rat antimesometrial decidual cell line. Endocrinology 1995;136:1913-1919.

Takahashi S, Reddy SV, Dallas M, Devlin R, Chou JY, Roodman GD. Development and characterization of a human marrow stromal cell line that enhances osteoclast-like cell formation. Endocrinology 1995;136:1441-1449.

Ubagai T, Lei K-J, Huang S, Mudd SH, Levy HL, Chou JY. Molecular mechanisms of an inborn error of methionine pathway: methionine adenosyltransferase deficiency. J Clin Invest, in press.

Patent

Chou JY, Lei K-J, Shelly LL. Glucose-6-phosphatase: the gene and protein and related mutations. Allowed May, 1995.

Current Protocol - HDB

Principal Investigator: Lawrence Charnas, M.D., Ph.D.
Heritable Disorders Branch, NICHD

TITLE:

93-CH-0112 - "The Treatment of Behavioral Disturbances in the Oculocerebrorenal Syndrome of Lowe: A Comparison Between Clomipramine and Desipramine and Placebo"

HYPOTHESIS: The behavioral disturbances in OCRL resemble those seen in Obsessive Compulsive disorders and will respond to modulation of the serotonin system through use of serotonin reuptake inhibitors.

STUDY DESIGN: A double blind, placebo controlled trial comparing the effects of clomipramine, a tricyclic antidepressant with relatively high serotonin reuptake inhibition with desipramine, a tricyclic antidepressant with a very similar side effect profile but 1% of the serotonin reuptake inhibition of clomipramine. Patients with OCRL whose behavioral profiles showed consistent obessional behaviors which were severe enough to warrant attempts at medical intervention were studied using 5 standard behavior rating scales during a two week placebo control period and then received 6 - 8 weeks of each active agent.

CURRENT RESULTS: Eleven subjects entered and completed the trial. Five of the 11 showed significant improvement in maladaptive behaviors and have been maintained on active agent through their local physician. In patients that responded to medication, one was clearly better than the other. Three patients withdrew due to side effects of increased agitation or aggressiveness, which resolved when active agents were discontinued. Desipramine was equally effective compared to clomipramine in improving maladaptive behaviors in OCRL. Side effect frequency was comparable among the two active agents. The study design with a ceiling of 20 subjects had inadequate power to distinguish between clomipramine and desipramine as more effective agents and was terminated in May, 1995.

CONCLUSIONS: Either clomipramine or desipramine appears more effective than placebo in improving the behavioral disturbances in OCRL. There is insufficient data to favor one agent over the other.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01106-12 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Continuity of Individual Differences in Reactivity in Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

S. J. Suomi	Head	LCE, NICHD
M. Champoux	Staff Fellow	LCE, NICHD
J. D. Higley	Staff Fellow	LCS, NIAAA
G. D. Byrne	IRTA Fellow	LCE, NICHD
D. Smucny	Pre-IRTA Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

NIAAA (Linnoila, Goldman); NIMH (Doudet); LABS (Mehlman, Taub); U of Puerto Rico (Phoebus); U of California, S.F. (Boyce); U of Minnesota (Gunnar)

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Comparative Behavioral Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates primate biobehavioral development through comparative longitudinal studies of rhesus and capuchin monkeys, with special emphasis on characterizing individual patterns of differential behavioral and physiological responses to environmental novelty and challenge and on determining long-term developmental consequences for individuals of different genetic backgrounds reared in different physical and social environments. During the past year prospective longitudinal studies of rhesus monkey infants differing in genetic pedigree but reared under identical nursery conditions revealed significant strain differences in blood CBC and serum chemistry, as well as differences in central serotonin metabolism and behavioral response to environmental challenge throughout the first 6 months of life. Other studies with rhesus monkey infants revealed significant effects of early social environmental factors on biobehavioral development in terms of alterations in neuroendocrine circadian rhythms and glucocorticoid sensitivity to routine caretaking activities to a far greater degree than previously presumed. Monkey infants reared under different social caretaking conditions also differed systematically in central serotonin metabolism from their first month onward; these differences were developmentally stable over the first 6 months. Field studies of rhesus monkey juvenile males showed that individuals with unusually low CSF levels of 5-HIAA, the primary central serotonin metabolite, exhibited unusually high levels of impulsive behavior, including unprovoked and escalating aggression. Most of these impulsive males were physically expelled from their natal troop well before puberty, with a very high subsequent mortality rate. Immunological studies of captive juvenile and adolescent rhesus monkeys demonstrated that low CSF 5-HIAA was associated with suppressed immunoreactivity, while PET scans of adolescent males with high vs. low CSF 5-HIAA levels (and low vs. high levels of unprovoked aggression) revealed significant differences in measures of regional cerebral glucose metabolism. Longitudinal studies of biobehavioral development in capuchin monkeys disclosed significant predictive relationships between early activity state profiles and later behavioral proclivities.

Project Description:

Objectives:

The project has five basic objectives: (1) to characterize individual differences among rhesus monkeys in their biobehavioral reactions to mild environmental challenges and to assess the long-term developmental continuity of such differences under standardized rearing conditions; (2) to determine and evaluate genetic and experiential factors that either alone or in concert influence the expression of such individual differences in response to challenge throughout development; (3) to describe the relationships among behavioral measures of relative reactivity and a variety of physiological concomitants, including patterns of hypothalamic-pituitary-adrenal activation, autonomic nervous system reactivity, monoamine metabolism, and immune system responsiveness; (4) to develop intervention strategies for altering ontogenic trajectories of individuals identified in early infancy as being at high risk for subsequent development of extreme reactions to species-normative environmental challenges; and (5) to assess the generality of findings from laboratory-born rhesus monkeys not only to wild populations of rhesus monkeys but also to capuchin monkeys (Cebus apella) and other primate species.

Methods Employed:

Rhesus monkey infants of known parentage are studied longitudinally in settings in which selected aspects of their physical and social environment are varied in systematic fashion. Behavioral and physiological data encompassing multiple levels of analysis are routinely gathered from each subject at various points under predetermined environmental conditions from birth to senescence. These data include frequent time-sampled focal animal observations utilizing sophisticated category-based scoring systems, neurological and neonatal reflex tests administered during infancy, periodic sampling of various hormones, neurotransmitters, and their metabolites in saliva, plasma, and CSF under carefully controlled environmental conditions, and systematic telemetric monitoring of psychophysiological activity in different physical and social settings. Some subjects receive occasional PET scans under isoflurane restraint, in collaboration with the NIMH imaging team. Subsets of these measures are also being obtained in longitudinal studies of capuchin monkeys.

The category-based observational scoring systems employ minicomputer-based data acquisition recorders that preserve the frequency, duration, and sequence of focal subject behaviors encompassed by each category, additionally recording the identity and concurrent actions of all interactive partners within the subject's social group. Blood samples are obtained via saphenous venepuncture under ketamine anesthesia or during brief (less than 30 seconds) physical restraint, then immediately spun down to obtain plasma, which is then frozen and stored for subsequent assay. Assays for plasma cortisol, ACTH, and CRF are performed under NICHD contract with Hazelton Laboratories (Vienna, VA). CSF samples are obtained under ketamine anesthesia via cisternal taps no more frequently than once per week (twice per month and 6 times per year total) from any one monkey. CSF samples are frozen immediately following collection and stored for subsequent assay for NE, MHPG (an NE metabolite), HVA and DOPAC (dopamine metabolites), and 5-HIAA (a serotonin metabolite). All assays are performed by collaborating researchers in the LCS, NIAAA. Heartrate patterns are monitored via a telemetric system covered by a monkey "jacket" that allows subjects unrestrained movement and behavior in both their home and test environments.

Major Findings:

During the past year several sets of new findings have identified both genetic and experiential factors that contribute to individual differences in behavioral and physiological development among rhesus monkeys of different ages, backgrounds, and current physical and social environments. Striking differences in blood CBC, serum chemistry, central serotonin metabolism, and behavioral (but not adrenocortical) response to brief social separation were found between rhesus monkeys infants whose parents came from wild populations in China and infants whose lineage could be traced back to rhesus monkey troops living in Northern India. Most of

these differences were apparent by 2 months of age and either remained stable or increased in magnitude in the ensuing months. These significant strain differences augment previous findings of significant differences in neonatal reflexes, activity patterns, and temperament during the first months of life between Chinese-derived and Indian-derived infants. Because both the Chinese-derived and Indian-derived infants were separated from their biological mothers at birth and were hand-reared under identical conditions in the LCE's neonatal nursery, these differences could not be attributed to differential postnatal experiences.

On the other hand, powerful effects of differential early experience were clearly demonstrated in longitudinal studies of infants reared in the neonatal nursery with surrogates and exposed to peers beginning at 1 month vs. those reared by their biological mothers. Nursery-reared infant exhibited a phase-shifted circadian rhythm for cortisol secretion relative to that of mother-reared infants, which in turn matched that of juveniles, adolescents, and adults in the LCE colony. Nursery-reared infants also demonstrated extreme glucocorticoid sensitivity, as indexed by sharp increases in salivary cortisol, to routine caretaking and husbandry procedures previously assumed to be relatively innocuous. These finding suggest that monkey mothers play an important role in buffering their infants from physiologically arousing effects of environmental perturbations during the initial postnatal weeks.

Early rearing condition differences were also disclosed for central serotonin metabolism. Nursery-reared infants had significantly lower CSF concentrations of 5-HIAA, the primary serotonin metabolite, than their mother-reared counterparts as early as 15 days of age, and these differences remained stable over the next 6 months. Because previous longitudinal research in the LCE has demonstrated long-term stability of individual differences in 5-HIAA concentrations from late infancy to early adulthood among monkeys maintained in comparable settings, the present findings suggest that these early experience effects on central serotonin metabolism may actually be life-long.

The consequences of large and stable individual differences in central serotonin metabolism for young male rhesus monkeys were explored in a study of free-ranging rhesus monkey troops living on Morgan Island, SC. The vast majority of rhesus monkey males living in the wild emigrate from their natal troop around or shortly after the time of puberty, usually in their fourth or fifth year; the males then typically join all-male "gangs" for a few months before finally working their way into a different troop. As in previous laboratory and field studies, individual differences in CSF 5-HIAA concentrations were associated with specific behavioral tendencies: juvenile males with low 5-HIAA concentrations were far more likely to exhibit impulsive behavior, unprovoked and escalating aggression, and inappropriate social responses (e.g., failure to defer to an obviously dominant individual) than their peers with moderate or high 5-HIAA concentrations. A high proportion of these low 5-HIAA juvenile males were physically expelled from their natal troop during their second and third year of life, far earlier than the species norm for age of male emigration. Virtually none of these early-expelled males successfully joined another social group, and a high proportion perished within a year, prior to the onset of puberty. Thus, among free-ranging rhesus monkey troops, unusually low CSF 5-HIAA in juvenile males not only is associated with impulsivity, inappropriate aggression, and social incompetence but also is a strong risk factor for premature emigration, subsequent solitary living, and early death.

Laboratory studies of immune functioning in captive adolescent (both male and female) rhesus monkeys revealed another risk associated with low CSF 5-HIAA concentrations. Monkeys with the lowest 5-HIAA concentrations also had the lowest T-cell and NK cell activity, as well as the highest white blood cell, mean corpuscular hemoglobin, and eosinophin counts. Imaging studies were also carried out on captive adult male rhesus monkeys who differed systematically in CSF concentrations of 5-HIAA and in behavioral ratings of impulsivity and aggressiveness. PET scans administered under isoflurane anesthesia revealed that monkeys with the highest rates of brain glucose metabolism also had the highest impulsive/aggressive ratings and the lowest CSF 5-HIAA concentrations. Regional analyses revealed even stronger correlations for orbital frontal cortex (both right and left), caudate (both right and left), left (but not right) amygdala, and right thalamic regions. In addition, low 5-HIAA (high impulsive/aggressive) monkeys required more barbiturate to induce anesthesia than did their higher 5-HIAA (lower impulsive/aggressive) counterparts.

Finally, normative longitudinal studies of capuchin monkey biobehavioral development this past year focused on the relationship between early measures of infant state and subsequent patterns of behavioral ontogeny and response to brief (1-hr.) social separation at 6 and 12 months of age. Early state measure were significantly correlated with both 6 and 12 month separation behaviors, but not behavioral patterns observed during baseline periods of group living. These findings regarding predictive relationships between early activity patterns and subsequent response to environmental challenge, but not to normative activities in capuchin monkeys mirrors extensive previous findings in rhesus monkeys.

Significance to Biomedical Research and the Program of the Institute:

Delineating and understanding the ways in which genetic and environmental factors interact to shape the course of an individual's ontogeny represents perhaps the most basic challenge in the study of developmental processes in whole organisms. The significance of the research encompassed by this project lies in its focus on both genetic and environmental sources of variance, its use of multiple longitudinal measures of both behavioral and physiological processes representing several levels of analysis, and its emphasis on early detection of extreme cases and subsequent strategies for intervention, using advanced nonhuman primate subjects who share many aspects of early behavioral and physiological development with human infants but who grow to maturity in only a fraction of the time required for comparable human development.

Proposed Course of Project:
In the coming year we plan to continue our longitudinal studies of rhesus monkey infants selectively bred for high vs. low CSF 5-HIAA levels and reared in different social environments, as well as initiating a new longitudinal study of infants selectively bred for extremes of 5-HIAA functioning and adrenocortical reactivity and subsequently reared differentially. In addition, the long-term developmental consequences of differential early experience with environmental controllability will be studied prospectively in a new cohort of selectively bred and differentially reared rhesus monkey infants. Studies of the interrelationships of various behavioral measures and physiological indices will continue, including direct experimental manipulations of subsets of these variables under carefully controlled laboratory conditions, as well as generalization studies in free-ranging populations of rhesus monkeys living in the wild. Finally, we will continue our prospective longitudinal investigations of biobehavioral development and response to environmental novelty and challenge in the LCE's capuchin monkey colony.

Publications:

Boyce WT, Champoux M, Suomi SJ. Salivary cortisol in nursery-reared rhesus monkeys: Interindividual stability, reactions to peer interactions, and altered circadian reactivity. Develop Psychobiol 1995;28:257-267.

Boyce WT, Higley JD, Jenerin J, Champoux M, Suomi SJ> Tympanic temperature asymmetry and stress behavior in rhesus monkeys and children. Arch Ped Adol Med, in press.

Byrne GD, Suomi SJ. Activity patterns, social interaction, and exploratory behavior in Cebus apella infants from birth to 1 year of age. Am J Primatol 1995;35:255-270.

Champoux M, Boyce WT, Suomi SJ. Biobehavioral comparisons between adopted and non-adopted rhesus monkey infants. Devel Behav Ped 1995;16:6-13.

Champoux M, Kriete MF, Higley JD, Suomi SJ. CBC and serum chemistry differences between Indian-derived and Chinese-Indian hybrid rhesus monkey infants. Amer J Primatol., in press.

Champoux M, Suomi SJ, Schneider ML. Temperamental differences in captive Indian- and Chinese-derived rhesus macaque neonates. Lab Anim Sci 1994;44:351-357.

Del Carmen R, Huffman LC. Epilogue: Bridging the gap between research on attachment and psychopathology. J Consult Clin Psychol, in press.

Doudet D, Hommer D, Higley JD, Andreason PJ, Moneman R, Suomi SJ, Linnoila M. Cerebral glucose metabolism, CSF 5-HIAA, and aggressive behavior in rhesus monkeys. Am J Psychiat, in press.

Higley JD, King ST, Hasert MF, Champoux M, Suomi SJ, Linnoila M. Stability of interindividual differences in serotonin function and its relationship to severe aggression and competent social behavior in rhesus macaque females. Neuropsychopharm, in press.

Higley JD, Mehlman PT, Poland RE, Faucher I, Taub DM, Vickers J, Suomi SJ, Linnoila M. Correlations of CSF 5-HIAA and CSF testosterone with different types of aggressive behaviors in a nonhuman primate model. Biol Psychiat, in press.

Higley JD, Suomi SJ. Reactivity and social competence affect individual differences to severe stress in children: Investigations using nonhuman primates. In: Pfeffer CR, ed. Intense stress and mental disturbance in children. New York: American Psychiatric Press, in press.

Higley JD, Suomi SJ, Linnoila M. Progress toward developing a nonhuman primate model of alcohol abuse and alcoholism. Soc Sci Med, in press.

Huffman LC, Del Carmen R, Bryan YE, Pedersen FA. Cry acoustics and temperament. In: Lester BM, Newman JD, Pedersen FA, eds. Biological and social aspects of infant crying. New York: Plenum Press, in press.

Kriete MF, Champoux M, Suomi SJ. Development of iron deficiency anemia in infant rhesus monkeys: A potential model for iron deficiency anemia in human infants. Lab Anim Sci 1995;45:11-15.

Mehlman PT, Higley JD, Faucher I, Lilly AA, Taub DM, Vickers J, Suomi SJ, Linnoila M. CSF 5-HIAA concentration are correlated with sociality and the timing of emigration in free-ranging primates. Am J Psychiat, in press.

Mehlman PT, Higley JD, Faucher T, Lilly AA, Taub DM, Vickers J, Suomi SJ, Linoilla M. Low cerebrospinal fluid 5-hydroxyindoleacetic acid concentrations are correlated with severe aggression and reduced impulse control in free-ranging nonuman primates (Macaca mulatta). Am J Psychiatr 1994;151:1485-1491.

Suomi SJ. Influence of Bowlby's Attachment Theory on research on nonhuman primate biobehavioral development. In: Goldberg S, Muir R, Kerr J, eds. Attachment theory: Social, developmental, and clinical perspectives. Hillsdale NJ: The analytic Press, in press.

Suomi SJ. Touch and the immune system. In: Field T, ed. The power of touch. Hillsdale NJ: Erlbaum, 1995;89-104.

Suomi SJ. Social and biological pathways that contribute to variations in health status: Evidence from primate studies. In: Properity, health, and well-being (Proceedings of the 11th Honda Foundation Discoveries Symposium). Toronto: CIAR Press, 1994;105-112.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01107-12 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Adaptation of Laboratory Reared Monkeys to Field Environments

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

S. J. Suomi	Head	LCE, NICHD
A. Timme	Fogarty Fellow	LCE, NICHD
G. Westergaard	IRTA Fellow	LCE, NICHD
P. O. Wagner	Research Psychologist	LCE, NICHD
C. Price	Biologist	LCE, NICHD
K. L. Rasmussen	Senior Staff Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

Caribbean Primate Research Center (Berard); New England Regional Primate Center (Jorgensen); SUNY-Buffalo (Berman); U Colorado (Laudenslager), U Florida (Boinski); U Massachusetts (Novak); Istituto di Psicologia, CNR (Visalberghi)

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Comparative Behavioral Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates how rhesus monkeys and other nonhuman primate species born and raised under different laboratory conditions adapt to placement into environments that contain specific physical and social features of the monkeys' natural habitat. Adaptation is assessed by examining behavioral repertoires and by monitoring a variety of physiological systems in these subjects, yielding broad-based indices of relative physical and psychological well-being. The responses of subjects to experimental manipulations of selected features of their respective environments are also assessed in similar fashion. Whenever, possible field data are collected for appropriate comparisons. An additional focus is on investigating the cognitive, behavioral, and social processes involved in adaptation to new settings or circumstances. Capuchin monkeys are employed in many of these studies because they are unique among monkey species in their propensity to manufacture and use tools to modify their physical environment.

During the past year new studies of spacial memory capabilities demonstrated that rhesus monkeys of all ages are exquisitely sensitive to the appearance of novel stimuli within a stimulus-rich enviroment, be they in captive or free-ranging settings. A study of male troop transfer under captive circumstances was initiated, while a field study comparing both behavioral and physiological characteristics of adult males who had joined new troops after natal troop emigration with those who remained solitary after emigration was completed. Long-term longitudinal analyses of mother-infant interactions in the same field setting uncovered a striking dynamic relationship between troop demographics and patterns of maternal care. Cross-species comparisons of mother-infant relationships between rhesus monkeys and Barbary macaques revealed interesting differences between these closely related macaque species in analogous field settings. Finally, extensive studies with capuchin monkeys extended the known range and complexity of their tool-using capabilities, including some previously thought to be exclusive to humans and their hominid ancestors.

Project Description:

Objectives:

The basic objectives of this long-term project are: (1) to study the process of adaptation to naturalistic physical environments in rhesus monkeys born and reared under laboratory conditions, (2) to determine the extent to which the observed process of adaptation generalizes to rhesus monkeys with different backgrounds and current home environments and to other primate species, (3) to assess the effects of experimental manipulations of selected features of the monkeys' captive environment in order to simulate specific phenomena characteristic of their natural habitats, and (4) to utilize these data to develop laboratory environments that are explicitly designed to foster various aspects of psychological well-being for their respective primate inhabitants, as well as to provide a means for assessing their capabilities for cognitive, behavioral, and social adaptation.

Methods Employed:

This project centers on longitudinal study of a group of 22-year-old rhesus monkeys and 4 generations of their offspring, all of whom live year-round in a 5-acre outdoor enclosure with shelter on the grounds of the NIHAC. The group-founding 22-year-old monkeys were born and raised under laboratory conditions without exposure to any conspecifics besides themselves; the same is true of their offspring. A comparison group of rhesus monkeys born and reared in the same laboratory as the founding study group subjects, but whose members have spent the intervening years living in indoor pens at the University of Massachusetts, Amherst, is now involved in parallel studies and data collection protocols, as are other long-term social groups of rhesus monkeys, some living in indoor-outdoor laboratory pens and some living in the field.

Time-sampled observations utilizing sophisticated behavioral scoring systems provide the primary mode of data collection for monkeys living in the 5-acre field enclosure at the NIHAC, and similar data are available from the various comparison groups. Monkeys in these groups are each observed for 10-minute periods of focal animal data collection several times each week by specially trained LCE personnel. During each observation period the frequency, duration, and sequence of the focal subject's activities are recorded according to a exhaustive category scoring system; the partners in each social interchange involving the focal subject are also identified, as is the nature and sequence of the partners' behavior during the interaction. Additionally, the location of all other group members is recorded at periodic intervals throughout each focal animal's observation period. The resulting data set is then entered into the basic longitudinal data base that has been maintained on each group member since birth. Appropriate files from this data base can be utilized to address specific empirical questions concerning longitudinal behavioral development and adaptations within and between the respective study groups and to compare these monkeys' patterns of environmental adaptation with various conspecifics having different backgrounds and different current living environments. In addition, new techniques for telemetric collection of psychophysiological data from the rhesus monkeys living in free-ranging environments have been developed and formal data collection continued this past year. Nonobtrusive techniques for obtaining neurohormonal data are also being developed for use on the free-ranging monkey groups. Techniques for using foraging patterns to assess memory functions and resource utilization under standardized conditions are being tested in different settings.

Finally, because capuchin monkeys are unique among monkey species in their propensity to use tools to modify their physical environment, systematic investigations of object manipulation and the development of tool use are carried out in some of the capuchin groups. In addition, tests of sophisticated cognitive and perceptual-motor capabilities are conducted within the capuchin monkeys' home environments. In these tests the monkeys first learn to operate a joystick that moves a cursor on a video display. Software has been developed to present subjects with a variety of tasks tapping different perceptual-motor and cognitive capabilities that can be "solved" via appropriate movement of the cursor; solutions to individual trials are rewarded via delivery

of a food pellet. This apparatus is also being utilized in comparative studies with rhesus monkeys and chimpanzees.

Major Findings:

During the past year extensive construction was continued at the LCE's 5-acre outdoor field station, limiting the access of the main study troop of rhesus monkeys to various sections of their 5-acre habitat during much of the year. Nevertheless, it was possible to complete the formal data collection for a study investigating spacial memory capabilities and differential responses to novel objects placed in different parts of the monkey's habitat. Comparable data were also gathered on rhesus monkeys living under different captive conditions. Initial analyses revealed highly similar patterns of findings across the different groups of subjects and environments, including age-sex differences in responsiveness to changes in stimulus array, as well as a strong tendency in all the monkeys to instantly identify novel objects in an otherwise familiar stimulus array and a less robust tendency to spot changes in the precise location of familiar objects.

Ongoing construction of the new shelter facility for the LCE field station necessitated a change in the interior fencing layout within the 5-acre enclosure. This change actually facilitated pilot testing of a "semi-permeable" enclosure system designed to permit simulation of naturalistic adolescent male troop emigration/transfer phenomena. The pilot testing clearly demonstrated that most aspects of emigration/transfer could be nicely simulated with minimal physical risk to the adolescent males, not a minor consideration given the high mortality associated with adolescent male emigration/transfer in the wild. The transfer patterns that emerged in the pilot testing suggested a number of interesting relationships between troop dynamics and demographics and the behavior of the emigrating/transferring adolescent males. These relationships are readily subject to rigorous empirical test, and we plan to begin doing so in the coming year.

A field study of long-term consequences of differential male emigration patterns in rhesus monkey troops on Cayo Santiago island (PR) was completed in FY95. In this study adult males 10-20 years of age who had failed to join another troop following emigration from their natal troop at 4-5 years were compared on a variety of physical, physiological, and behavioral measures with age-matched males who had joined and remained in other troops following their own natal troop emigration. The initial analyses revealed remarkably few obvious differences between solitary and social males in their psychophysiological and hormonal profiles: if anything, solitary males tended to be heavier, had more body fat, and a less labile pattern of testosterone response to brief capture. These results indicate that solitary males that survive their first few years after natal troop emigration seem remarkably well-adapted to their solitary status and do not appear to be at extreme physical risk for mortality or morbidity, unlike males who emigrate prior to puberty. The behavioral data are currently being analyzed.

Other longitudinal data collected from the wild rhesus monkey troops on Cayo Santiago investigated the relationship between long-term changes in troop demographics and maternal caretaking patterns. A complex dynamic relationship with multiple feedback patterns emerged from the analysis: When troop size is relatively small, mothers tend to be laissez-faire with respect to their infants' interactions with nonkin troop members, and as the infants mature, their interactions with nonkin continue to be extensive and mostly positive. However, as the troop size increases, mothers become increasingly restrictive of their infants' activities, largely limiting their interactions with nonkin. As these infants mature, their interactions with nonkin remain relatively infrequent and become increasingly agonistic. If this trend continues over successive generations, the troop eventually splits up, fissioning along kinship lines. The resulting "fragment" troops are much smaller than the original troop, and within these fragment troops mothers once again tend to be laissez-faire toward their infants, starting the above cycle anew. Thus, troop demographics affect maternal restrictiveness, which in turn affects troop demographics, etc.

Patterns of maternal care exhibited by the free-ranging rhesus monkey mothers on Cayo Santiago were compared in another study with maternal care patterns exhibited by a closely related species, Barbary macaques (M. silvanus), also living in a free-ranging, provisioned environment. Relative to Barbary macaque mothers, rhesus monkey mothers were generally less restrictive (i.e., let their infants explore and interact with nonkin more frequently) during the initial weeks of life, but during succeeding months this species difference was reversed. These findings appear consistent with species differences in the types of threats" infants typically encounter as they grow up: Barbary macaque infants are at risk to be "kidnapped" by nonkin females during their first month of life, an exceeding rare occurrence in rhesus monkey troops. On the other had, rhesus monkey juveniles are more likely to be attacked by nonkin group members after their initial months than are Barbary macaques, who are generally much less aggressive as a species. Thus, mothers in these two species adjust their maternal care to counter the specific types of risks that their infants are most likely to experience at different stages of development.

Extensive research investigating the tool-using capabilities of capuchin monkeys was continued in FY95, considerably expanding the known range and complexity of tool-using tasks the species is able to master. In addition to continued use of sticks, bamboo, and bones as probing tools and stones and bamboo as cutting tools, individual capuchins learned how to use a pestle, employed a variety of sticks, stones, bamboo pieces, and iron and copper fragments as digging tools, and developed a simple lithic technology. Most of the successful tool-using monkeys exhibited a significant lateral bias in their manipulation of the tools. The same individuals also displayed the strongest lateral bias in eye dominance and rotational manipulation, and they were also the monkeys most likely to use a mirror to guide their manipulation of objects hidden from direct view. Many of the tasks mastered by these monkeys were previously thought to exceed the capabilities of all primates other than chimpanzees, humans above the age of 2 years, and our immediate hominid ancestors. The implications of these new findings for understanding the emergence of tool-use in proto-human culture are considerable.

Finally, data from several field studies investigating the relationship between social structure, environmental context, and specific vocal patterns in several species of New World monkeys were published in FY95.

Significance to Biomedical Research and the Program of the Institute:

The discovery that laboratory-born-and-reared rhesus monkeys readily adapt to naturalistic habitats and retain species-normative behavioral repertoires and patterns of social organization in the absence of any exposure to wild-born, ferally experienced adult conspecifics is of considerable significance in its demonstration that such complex behavioral capabilities and characteristics are clearly preserved in the genetic heritage of members of an advanced nonhuman primate species, even after many generations of laboratory living. Additionally, the relative ease, speed and thoroughness of species-normative adaptation to a naturalistic environment shown by these rhesus monkeys should encourage primate conservation efforts based on the establishment of captive breeding colonies with subsequent introduction into protected naturalistic habitats. They also provide a basis for establishing scientifically rigorous standards regarding the creation of laboratory environments that "promote the psychological well-being" for captive primate subjects, as has recently been mandated by statute through an amendment to the Animal Welfare Act. The demonstration of sophisticated tool-using capabilities in capuchin monkeys provides evidence for continuity between these monkeys and apes and humans in several important dimensions and serves as a basis for focused investigation of cognitive processes and neurobehavioral correlates that underlie the use of tools in these diverse species. Finally, the development of procedures that promote or enhance well-being in nonhuman primate subjects growing up in captive environments can provide insights regarding aspects of physical and social environments that serve to promote or enhance well-being in human infants, children, and the elderly.

Proposed Course:

We plan to continue detailed longitudinal observations of the rhesus monkey troop living in the 5-acre outdoor enclosure at the NIHAC and on the comparison group living indoors at the University of Massachusetts. We also plan to continue longitudinal data collection on the free-ranging rhesus monkey troops living on Cayo Santiago. Additional studies of complex tool use in capuchin monkeys will investigate the basis for the dramatic individual differences in tool-using capabilities within the LCE's capuchin colony, as well as identifying neurobiological correlates that might underlie such differences.

Publications:

Boinski S. Stress responses in primates: Proximate mechanisms in the evolution of social organization. In: Foster SA, Endler JA eds. Geographic variation in behavior: An evolutionary perspective. Oxford: Oxford University Press, in press.

Boinski S. Affiliation patterns among male Costa Rican squirrel monkeys. Behaviour 1994;130:191-209.

Boinski S, Moraes E, Kleiman D, Deitz J, Baker A. Intra-group vocal behaviour in wild golden lion tamarins (Leontophithecus rosalia): Honest communication of individual activity. Behaviour 1994;130:53-76.

Boinski S, Campbell AF. Use of trill vocalizations to coordinate troop movement among white-faced capuchins: A second field test. Behaviour, in press.

Boinski S, Mitchell CL. Male residence and association patterns in Costa Rican squirrel monkeys (Saimiri oerstedi). Am J Primatol 1994;34:157-170.

Boinski S, Mitchell CL. Wild squirrel monkey (Saimiri sclureus) "caregiver" calls: Contexts and acoustic structure. Am J Primatol 1994;35:129-138.

Clarke AS, Boinski S. Temperament and reactivity in primates. Am J Primatol, in press.

Friedman EM, Boinski S, Coe CL. Interleukin-1 induces sleep-like behavior and alters call structure in juvenile rhesus macaques (Macaca mulatta). Am J Primatol 1995;35:143-155.

Jorgensen MJ, Suomi SJ, Hopkins WD. Using a computerized testing system to investigate the pre-conceptual self in primates and humans. In: Rochet P ed. The self in early infancy: Theory and research. Amsterdam: North Holland-Elsevier Press, in press.

Novak MA, Rulf A, Munroe H, Parks K, Price CM, O'Neill P, Suomi SJ. Using a standard to evaluate the effects of environmental enrichment. Lab Anim 1995;24:37-43.

Samerooff AJ, Suomi SJ. Primates and persons: A comparative developmental understanding of social organization. In: Cairns RB, Elder GH, Costello EJ, McGuire A eds. Development science. New York: Plenum Press, in press.

Suomi SJ. Nonverbal communication in nonhuman primates: Implications for the emergence of culture. In: Molnar P, Segerstrale U eds. Where nature meets culture: Nonverbal communication in social interactions. Hillsdale NJ: Lawrence Erlbaum, in press.

Suomi SJ, Novak MA, Well A. Aging in rhesus monkeys: Different windows on behavioral continuity and change. Develop Psychol, in press.

Timme A. Sex differences in infant integration in a semi-free ranging group of Barbary macaques (Macaca sylvanus) at Salem, FRG. Am J Primatol, in press.

Westergaard GC. The subsistence technology of capuchin. Int J Primatol 1994;15:899-906.

Westergaard GC. Current perspectives in primate laterality. Am J Primatol 1994;35:327-330.

Westergaard GC. The stone-tool technology of capuchin monkeys: Possible implications for the evolution of symbolic communication in hominids. World Archeol 1995;27:1-9.

Westergaard GC, Greene JA, Babitz MA, Suomi SJ. Pestle use and modification by tufted capuchins (Cebus apella). Int J Primatol 1995;16:643-651.

Westergaard GC, Hyatt CW. The responses of bonobos (Pan paniscus) to their mirror images: Evidence of self-recognition. Hum Evol 1994;9:273-279.

Westergaard GC, Suomi SJ. The use of probing tools by capuchin monkeys: Evidence for increased right-hand preference with age. Int J Primatol 1994;15:521-529.

Westergaard GC, Suomi SJ. A simple stone-tool technology in monkeys. J Hum Evol 1994;27:399-404.

Westergaard GC, Suomi SJ. Aimed throwing of stones by tufted capuchin monkeys (Cebus apella). Hum Evol 1994;9:323-329.

Westergaard GC, Suomi SJ. Stone-tool bone surface modification by monkeys. Current Anthropol 1994;35:468-470.

Westergaard GC, Suomi SJ. The manufacture and use of bamboo tools by monkeys: Possible implications for the development of material culture among east Asian hominids. J Archeolog Sci, in press.

Westergaard GC, Suomi SJ. Assymetrical manipulation in the use of tools by tufted capuchin monkeys (Cebus apella). Folia Primatol 1994;63:96-98.

Westergaard GC, Suomi SJ. The production and use of digging tools by monkeys: A nonhuman primate model of a hominid subsistence activity. J Anthropol Res 1995;51:1-8.

Westergaard GC, Suomi SJ. Mirror inspection varies with age and tool-using ability in tufted capuchin monkeys (Cebus apella). Hum Evol, in press.

Westergaard GC, Suomi SJ. The stone artifacts of capuchins (Cebus apella). Int J Primatol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HD 01112-09 LCE

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Home and Out-of-Home Care on Child Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	H. Wessels	Visiting Fellow	LCE, NICHD
	K.J. Sternberg	Research Psychologist	LCE, NICHD

COOPERATING UNITS *(if any)*
Center for Human Growth and Development, U of Michigan (Bookstein); Institute of Psychology, University of Goteborg (Broberg, Hwang); Institute for Applied Socialization Research, Berlin (L. Ahnert); S. Viernickel.

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Section on Social and Emotional Development

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves a longitudinal study conducted in Sweden examining the effects of center day care, family day care, and home care on the development of 145 children recruited at an average of 16 months of age. Initial multivariate analyses indicated that type of care had no reliable impact on child development, but that the quality of home care and the quality of alternative care have substantial effects on the children's verbal abilities, their social skills, and their behavioral attitudes. Subsequent analyses of data gathered shortly after the children started school indicated that the development of verbal and mathematical abilities as well as the development of personal maturity (as measured by field-independence, ego-control, and ego-resilience) were affected by the varying child-care histories the children experienced since their first enrollment in the study. These analyses indicated that family daycare has modest but significant negative effects on both verbal abilities and personal maturity, whereas center-based care has beneficial effects in both domains. The effects of the quality of home and alternative care appeared to diminish as the children moved into the formal educational system. Plans are now being developed for assessment of these children at 14-15 years of age. In a related study, SSED staff are assessing the psychophysiological and behavioral tendencies of young infants at home in order to assess predictions about the effects on adaptation to out-of-home care.

Project Description:

Objectives:

Since the establishment of the SSED in July 1987, staff have been involved in the analysis of data from a longitudinal study in Sweden examining the effects of center daycare, family daycare, and home care on social, personality, and intellectual development. Analyses have been designed to explore the origins and temporal course of individual differences among the children using novel analytic techniques that have been developed for such multi-measure longitudinal studies.

Guided by previous research in the USA, staff expected that type of care (home care, center daycare, or family daycare) would have no significant impact on aspects of child development but that the quality of home and out-of-home care, the child's temperament, and his/her initial patterns of sociability would predict individual differences in later indices of social and intellectual competence. In addition, this data set has been used to develop composite measures of childhood aggression and noncompliance so that staff could explore how enrollment in daycare and quality of care affected the development of aggression and compliance in children. In the past year, analysis of data gathered after entry in the public schools has begun. Analyses to date have focused on two of the most important domains of child development, cognition and personal maturity.

Recently, SSED staff and collaborators undertook a longitudinal study of the physiological and behavioral correlates and predictors of adaptations to out-of-home care in infancy. In this study, 50 infants in Berlin are being assessed immediately prior to and after enrollment in day care centers.

Methods Employed:

145 children on the waiting lists for childcare facilities in Sweden were enrolled in the Swedish Longitudinal Study at an average of 16 months of age. In an initial visit to the homes, data were collected about family background, social networks, care arrangements, relative parental involvement, temperament, and the child's initial response to the visiting adult. In a second visit, the HOME inventory (Caldwell, 1970) and the Belsky and Walker (1980) observation checklist were employed to assess the quality of the home environment. Children enrolled in center-based daycare or family daycare entered these settings within two weeks of these first assessments. Six weeks later their childcare facilities were visited by a member of the research staff to obtain observational and interview data about the care arrangement and its quality.

One and two years thereafter the families were visited again (waves two and threee of data collection. Durings these visits, stranger sociability was assessed, and the parents were questioned about their care arrangements. In the second visit, the quality of the home environment, paternal involvement in child care, and the children's personal maturity and peer play skills were measured. Trained psychologists administered scale C of the Griffiths Developmental Scales in order to assess the verbal abilities of children in both phases. Again, for the children enrolled in family or center-based daycare, these facilities were visited to collect data about the quality of the care facility and the children's behavior in the alternative care setting.

The fourth and fifth waves of data collection took place prior to enrollment in elementary school and when the children were in second grade. During these visits, the children's verbal and mathematical abilities were tested by trained assistants and data about paternal involvement, the children's personal maturity, peer play skills, and current care arrangements were gathered. Additionally, data about the quality of the preschool and alternative care settings were collected again. In phase five, an intensive interview with the elementary school teacher was conducted in order to obtain additional information about the children's peer behavior, adjustment to school, cognitive and social skills, and personality.

In the new study, children's heartrate and salivary cortisol levels are being recorded at home as well as on several occasions following enrollment in day care. Responses to separation are also being observed and coded.

Major Findings:

In earlier reports from the Swedish Longitudinal Study, SSED staff confirmed that type of child care had no impact while quality of home care and the quality of alternative care had the most consistent and powerful effects on emergent social skills, personality maturity, and behavior in a task-like setting designed to elicit children's compliance. More recently, the association between child-care arrangements and subsequent cognitive and personality development has been examined. Parental ratings of field independence, ego-resilience, and ego-control obtained using the California Q-set when the children averaged 28, 40, 80, and 101 months of age revealed virtually no difference between the developmental trajectories of the children in home care and in center-based daycare. By contrast, ego-undercontrol decreased less, while ego-resilience and field independence increased less, in children who attended family daycare than in the children in either center care or exclusive parental care. The quality of home and out-of-home care, as well as socioeconomic status and family background, did not qualify these effects of the type of care experienced. Other analyses of the same data were designed to evaluate the Five Factor Model of personality. The factors identified by John et al. in a study of adolescents were barely reliable when the children averaged 29 months of age, but were sufficiently and increasingly reliable in the assessments which took place when the children averaged 40, 80, and 101 months of age. Results of a cross-validation, employing data obtained when the children were in second grade, indicated that the factors were meaningfully related to measures of the childrens' cognitive abilities and school adjustment.

The divergent developmental trajectories of the children in the home-care and the center-based daycare groups differed with respect to the development of verbal abilities as well. When the children were eight years old, and in the second grade of elementary school, tests and teachers' ratings of children's cognitive abilities were administered to the 123 children (65 girls) remaining in the study. Tested ability was related to the number of months children had spent in center-based day care before 3 1/2 years of age. Structural and dynamic aspects of the child care quality also predicted cognitive abilities among those children who had spent at least 36 months of their preschool years in out-of-home care. Both tested and rated cognitive abilities in second grade were related to earlier measures of verbal ability and to paternal involvement during the pre-school years.

Significance for Biomedical Research and the Program of the Institute:

This program of research has underscored the importance of quality of out-of-home care and the role of factors outside that care setting, such as the quality of home care and maternal characteristics, when evaluating the effects of daycare. Our research also underscores the value of longitudinal studies of adjustment and problem behaviors in different child-rearing settings. More generally, this research provides information about the factors that account for the emergence, maintenance, and alteration of individual differences among children. Such findings provide valuable basic evidence about developmental processes, while informing public policy concerning issues such as daycare and parental leave policies. Research on individual differences in patterns of adaptation promises to help better predict how different children will be affected by enrollment in day care.

Proposed Course of Project:

Ongoing analyses are designed to further explore longitudinal associations in several other domains, including the development of peer and social skills. Plans are also being made for further assessment of these children in the 8th and 9th grades at school. In the Berlin study, further subjects are still being recruited into the study, and data reduction is in process.

Publications:

Lamb ME. Daycare. In I Sigel, A Renninger (vol eds.), Child psychology in practice, W Damon (series ed.), Handbook of child psychology (fifth edition). New York: Wiley, in press

Prodromidis M, Lamb ME, Sternberg KJ, Hwang CP, Broberg AG. Aggression and noncompliance among Swedish children in center-based care, family day-care, and home care. Interna J Behav Develop, 1995; 18: 43-62

von Eye A, Kreppner K, Spiel C, Wessels H. Life events spacing and order in individual development. In TA Kindermann and J Valsiner (eds) Construction of self and social relationships: Combinatorial research in developmental psychology. Hillsdale, NJ: Erlbaum, 1995: 147-164

Wessels H. Betreuerverhalten und seine Bestimmungsfaktoren in Tageseinrichtungen für Kinder unter drei Jahren. In: W. Tietze (ed): Neue Entwicklungen in der Kleinkindpädagogik. Berlin: Luchterhand, in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01114-08 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Individual Differences in Physical and Affective Functioning in Infancy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	K. Sternberg	Research Psychologist	LCE, NICHD
	B. Leyendecker	Fogarty Fellow	LCE, NICHD
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LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.3	2.3	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project focuses on long-term consequences of individual differences in physical, social, and affective functioning and the description of early interaction in diverse ecological contexts. In one study, we studied infant-mother interaction and attachment in infants from upper-middle class Euro-Americans and lower-class Central American families, developing culturally-sensitive criteria for evaluating normative social behavior and development. Regardless of social or ethnic background, mothers spent comparable amounts of time in discrete activities such as feeding, caring for, and playing with their infants. Comparable observations of mothers and infants in Costa Rica, Quebec, Germany, the Central African Republic, and Colombia are designed to further explore the effects of culture and context on early interactions.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

Most students of behavioral development now agree that variations in developmental processes and outcomes are jointly determined by complex interactions between inherited dispositions and tendencies, on the one hand, and by experiential histories on the other. Empirically demonstrating such patterns of influence has proven difficult, however, because it demands the ability to conduct longitudinal studies incorporating sensitive measures of both biogenetic factors, developmental ecologies, and early interaction. One goal of the SSED staff has been to demonstrate these patterns of influence by undertaking detailed observational research on infants.

In one such multicultural investigation, SSED staff are developing techniques to describe the interaction between mothers and their infants in early life and to assess the effects of particular patterns of parenting, infant and maternal characteristics, and family patterns on subsequent attachment behavior on infants from divergent (and under-studied) backgrounds using both upper-middle class Euro-American (EA), lower-class Central American (CA) immigrants and groups of families in Costa Rica. It is believed that individual differences in the quality of prior interaction between mothers and children determine infants' attachments to their mothers and that the quality of attachment is a powerful predictor of the infant's psychosocial adjustment and behavior in the future. These associations have only been demonstrated in middle-class Caucasian populations, however. Little is known about patterns of infant-mother interaction in different ethnic and cultural subgroups, and it is not known whether similar predictive associations exist in non-mainstream populations. One of the component studies involves repeated observations of mother-child interaction to permit assessment of the influence of parenting styles on attachment and socioemotional development.

Methods:

For a study of Euro- and Central American mothers and infants in the Washington metropolitan area, mothers were contacted before their first-born infants were 15 weeks old. Four home visits took place when the children were three months of age, and other visits were made when the children were 4, 8 and 12 months old. When the infants were three months old, 4-hour observations of parent-infant interaction and infant behavior were scheduled throughout the day in overlapping units to ensure coverage of a 12-hour cycle. The four 3-month assessments had the same general format, incorporating age-appropriate measures for assessing maternal perceptions of infant temperament, measures of parental stress, social support, life stress, and marital relationships. The 4-, 8-, and 12-month assessments included observations of structured interaction such as feeding and teaching, as well as age-appropriate measures of infant temperament, including both maternal questionnaires and behavioral observations. The final 12-month assessment included laboratory observation of infant-mother attachment and social interaction.

Observational data were also gathered in Costa Rica and are being gathered in Germany, Quebec, Colombia and the Central African Republic. These samples were selected to represent equal proportions of subjects from at least two subsamples in each country. When the infants were three months old, they were observed throughout the day in overlapping units to ensure coverage of 12-

hour cycles, as was true in the USA.

Major Findings:

In our studies of the Euro-American and Central-American mothers in the Washington area, mother-infant interaction during the free play, teaching, and feeding situations at 4, 8, and 12 months of age were coded microanalytically. Individual differences on the discrete measures of infant behavior and behavioral coordination during bouts of mother-infant play were not stable and were related to Strange Situation behavior at some but not all points in time. Frequency measures of maternal behavior were not related to the infants' attachment status, but the responsivity scores were stable over time and strongly related to the quality of attachment. In both groups, mothers of secure infants scored around the middle, whereas mothers of resistant infants scored at either higher or intermediate levels. Mothers of avoidant infants tended to score lower in the Euro-American sample and higher in the Central American sample. Disorganized infants in both groups were less involved than secure infants in the tactile exploration of objects at all three ages. Central American mothers of disorganized infants consistently scored higher, whereas the Euro-American mothers of disorganized infants scored either lower or higher on the measures of responsivity. Didactic maternal behavior in the teaching situation decreased as the infants grew older, whereas task-oriented infant behavior increased with age. Individual differences in maternal teaching behavior were not stable over time, although the level of maternal vocalization was stable. Infants and mothers in the two samples differed with respect to discrete behaviors, but there were only minor differences between the samples with respect to levels of mutual coordination or the probability of secure attachment behavior in the Strange Situation. Frequency and duration measures of maternal and infant behavior in the teaching sessions were largely unrelated to later attachment status, but individual differences in indices of mutual coordination were significantly related to attachment security. A-classified dyads were characterized by coordinated attention on objects, C-classified dyads by coordinated attention on each other, and D-classified dyads by low scores on both coordination measures. Analysis of behavior in the feeding sessions has yet to be completed.

Initial analyses of data focused on the general patterning of everyday experiences in families comprising recent immigrants from Central America and in families comprising well-educated Euro-Americans. Descriptive analyses revealed remarkable similarities and few differences in the everyday experiences of infants in these two diverse groups. Both groups of mothers spent most of their time playing with, feeding, caring for, or holding their infants. Fathers spent little time with their infants during the day and their presence did not affect the amount of time spent in various social contexts, although significantly more holding occurred when the fathers were present. Other people spent more time with the infants in the Central American families and less time alone with the infants in the Euro-American families than fathers did. Mother and infant vocalizations and mutual attention occurred more frequently during play than in bouts of feeding or holding.

Subsequent analyses were focused on the influence of context on the observed levels of dyadic interaction, and on the influence of length of observation on the reliability of interactional measures. There were no group differences in the amounts of time spent by the mothers and infants in well-attuned states, or in different functional contexts, but the EA dyads spent more time in disharmonious states. Securely attached dyads in both samples spent more time in well-attuned states as well as in playful interactions rather than not interacting. Forty-five minute

blocks of observation yielded notably unreliable measures of individual differences but reliability increased considerably as the duration of the observations was expanded. The amounts of time spent in well-attuned and in disharmonious interactions varied substantially across contexts, with notable differences between the two groups. The results indicate that we can maximize subcultural differences by focusing on single contexts and minimize subcultural differences by averaging across a variety of naturally occurring contexts.

Mothers' reports of their daily activities when the infants in the Washington study were 8 months and 12 months of age were also analyzed. In this study, we explored the everyday experiences of 40 infants from families who migrated recently from Central America and of another 42 infants from middle class Euro-American families. Detailed descriptions of the previous 24 hours were obtained by interviewing the mothers and compiling recall diaries. The infants' experiences and activities were very similar in both groups, and the effects of the mothers', fathers', or others' presence on ongoing activities were similar, too. The groups differed with regard to (1) the circadian distribution of activities, (2) opportunites for interactions with various people, and (3) the differences between weekday and weekends. Overall, the social worlds of the Central American children were characterized by the simultaneous presence of several people and thus by multiple social partners. The social worlds of the Euro-American children were characterized by more opportunities for dyadic interactions and by exposure to fewer partners.

Similar observations of 20 three-month-old Costa Rican infants with middle class mothers and another 20 with working class mothers also revealed remarkable similarities and few differences in everyday experiences of infants in these two groups. Infants spent most of their time being cared for or held, and engaging in visual and verbal interaction. Fathers spent little time with their infants. When their fathers were present, middle class infants were more likely to be held and not to interact with anyone, whereas the working class infants were more likely to be fed. Regardless of social class, other people spent more time alone with the infants than fathers did. Mother and infant vocalizations and mutual attention occurred more frequently during play than in bouts of caring, feeding, or holding. There were few social class differences, although working class mothers spent more time feeding their infants than middle class mothers did.

Significance for Biomedical Research and the Program of the Institute:

The significance of our research lies in our focus on infants in diverse cultural contexts. By providing detailed observations and descriptions of culturally-specific parenting behavior and infant experiences in everyday life, the results extend our knowledge of normative social behavior. Exceptions to previously established associations between mother-infant interaction and infant-mother attachment may be revealed, which in turn could have important implications for the assessment of developmental processes. Since most studies involve families already assimilated into the mainstream culture, this project may disclose ethnocentric biases in our understanding of how environmental influences affect children's socioemotional development. Research on various subcultures affords the opportunity to obtain a more inclusive understanding of biobehavioral development.

Proposed Course of Project:

Analyses of interaction during the feeding sessions are now being conducted, as are analyses of the effects of functional context (i.e., feeding, caretaking, play) on measures of interactional harmony in dyads involving 3-month olds. Comparable data, using the same observational tools, have also been gathered in Germany, Colombia, Costa Rica, Quebec and the Central African Republic. These data will permit further insight into the existence and importance of divergent childcare practices.

Publications:

Fracasso MP, Lamb ME, Porges SW, Rosenberg AA. Cardiac activity in infancy: Reliability and stability of individual differences. Infant Behavior and Develop, 1994, 17: 277-284

Fracasso, MP, Lamb, ME, Schölmerich, A, Leyendecker, B. The ecology of mother-infant interactin in Euro-American and immigrant Central American families living in the United States. Internat J Behav Develop, in press

Haynie DL, Lamb ME. Positive and negative facial expressiveness in 7-, 10-, and 13-month-old infants. Infant Behav Develop, 1995; 18: 257-259

Lamb ME. Heredity, environment, and the question "why?" Behav Brain Sci, 1994; 17: 751

Lamb ME, Fracasso MP. Les dimensions der temperament: Physiologie, comportement et perceptions maternelles [The dimensions of temperament: Physiology, behavior, and maternal perceptions]. In R Tessier, GM Tarabulsy (eds) Enfance et famille: Contortes de developpements [Child and family: Contexts for development] (French). Quebec City: Les presses de l'Université Laval, in press

Lamb, ME, Nash, A, Teti, DM, Bornstein, MH. Infancy. In M Lewis (ed), Child and adolescent psychiatry: A comprehensive textbook (Second edition). Baltimore: Williams and Wilkins, in press

Leyendecker B. The influence of birth-order on opportunities for interactions between parents and their 10 week old infants. German J Psychol, in press

Leyendecker B, Lamb ME, Schölmerich A, Fracasso MP. The social worlds of 8- and 12-month-old infants: Early experiences in two subcultural contexts. Soc Develop, 1995; 4: 194-208

Posada G, Wu-Fang Gao Y, Haaland W, Sinevaag B, Schölmerich A. Q-sort descriptions of real and ideal children by mothers and experts. In E Waters, B Vaughn, G Posada, K Kondo-Ikemura (eds), Caregiving, cultural, and cognitive perspectives on secure-base behavior and working models: New growing points of attachment theory and research. Monog Soc Res Child Development, 1995; 60: Serial number 244

Schölmerich A. Process and result of manipulative exploration. In H Keller, K Schneider & B. Henderson (eds), Curiosity and exploration: Theoretical perspectives, research fields, and applications. Berlin, Heidelberg, New York: Springer Verlag, 1994: 241-257

Schölmerich A, Fracasso MP, Lamb ME, Broberg AG. Interactional harmony at 7 and 10 months

of age predicts security of attachment as measured by Q-sort ratings. Soc Develop, 1995; 4: 62-74

Schölmerich A, van Aken MAG. Attachment security and maternal concepts of ideal children in Northern and Southern Germany. Internat J Behav Develop, in press.

Schölmerich A, Leyendecker B, Keller, H. The cultural context of early infancy: Culture, communications, and eye-contact. In J. Valsiner (ed), Child development within culturally structured environments, Vol. 3: Comparative-cultural perspectives. Norwood, N.J.: Ablex, 1995: 29-50

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01115-08 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Domestic Violence and Evaluation of Children's Testimony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	K. J. Sternberg	Research Psychologist	LCE, NICHD
	Y. Orbach	IRTA Fellow	LCE, NICHD
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COOPERATING UNITS *(if any)* Hebrew U, Jerusalem (Greenbaum); Jerusalem Municipality (Lorey); U Rochester (Cicchetti); Ministry of Social Welfare, Jerusalem (Berger, Berkowitz, Eden, Hovav, Karniel); U. Haifa (Hershkowitz); U. NC, (M. Everson); U. Cincinnati, (Boat); Children's Hospital, San Diego (Chadwick; Landsverk); Darvish; Esplin; Larson; Yudilevitch.

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Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.2	1.7	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This program of research involves two projects. The goal of the first is to explore the effects of domestic violence on children who were either victims of physical abuse, witnesses of spousal abuse, both victims and witnesses, or neither victims nor witnesses. These children were first assessed in 1988/9 when they were between 8 and 12 years of age and the results of these assessments have been reported in previous annual reports. The children, their parents, their teachers, and their schools are now being reinterviewed. These assessments will be completed early in FY 96 and analyses of the data will then begin.

The goal of the second study is to assess the field reliability and validity of a system for eliciting and assessing statements by children who are alleged to have witnessed sexual abuse. Sites around the United States and in Israel are providing transcripts of audio- or videotaped interviews along with case files to establish case facts independent of the statements. The statements are being assessed using a revision of the Criterion-Based Content Analysis (CBCA) techniques, while case facts are independently rated using "ground truth" assessment scales developed by SSED staff. Other analyses are focused on the relationship between interviewer style and quality of information provided by the child. Several studies have confirmed that open-ended questions elicit longer and more detailed responses than more focused questions, regardless of the number of incidents experienced and the language (English or Hebrew) in which the interview was conducted. Recent studies have shown that interviewers can increase the length and richness of children's accounts by following SSED-designed protocols designed to enhance reliance or recall memory.

Project Description:

Objectives:

Effects of domestic violence: It appears self-evident that domestic violence has adverse effects on children, but there have been few methodologically sound studies of the nature, magnitude, and permanence of these effects. Before our research began, there had been no adequately controlled studies comparing the effects of witnessing and/or being the victim of domestic violence, no controlled investigations of the factors protecting some children and making others more susceptible to adverse effects, and no studies exploring children's phenomenological perceptions of domestic violence. The first project has been designed specifically to address these lacunae and to examine the extended effects of experiencing domestic violence by following the children's development longitudinally.

Children's testimony: The objective of the second project is to evaluate and improve upon the Criterion-Based Content Analysis (CBCA) system for eliciting and assessing statements made by children who are alleged to have been victims or witnesses of sexual abuse. The validity of the CBCA technique is being evaluated by comparing information from investigative case files with ratings on the CBCA criteria performed by coders blind to the status and background of the cases. Transcripts are also exhaustively coded to explore the relationship between interviewer utterance type and the richness and length of the responses elicited. These descriptive accounts have helped SSED staff to develop more effective interview techniques, which increase the length and richness of children's accounts.

Methods Employed:

Effects of domestic violence: The first study was undertaken in Israel because it was feasible to obtain a relatively large and methodologically appropriate sample of children in two-parent, violent families, whereas research in the United States often involves abused children from single-parent families and children who reside with their mothers in battered women's shelters. We also had excellent collaborators in the service agencies there, an essential prerequisite for methodologically and clinically sound research of this sort. Finally, because mobility in Israel is less than in the US, it was possible to examine the effects of domestic violence in a four-year longitudinal study. We currently plan to assess all children once again when they are enlisted for military service.

110 children, their parents, homeroom teachers, and peers initially provided information about the target children's functioning. The families, selected by the staff of the Ministry of Welfare in Jerusalem and Tel Aviv, Israel, were divided into four groups: children who experienced chronic physical abuse by a parent, with at least one incident occurring 6 months prior to the interview; children who witnessed chronic spouse abuse; children who both witnessed and were victims of parental abuse; and matched controls who experienced no domestic violence. Cross-validation of group classification was determined through interviews with parents and children.

Parents provided demographic data and described their children using the Dimensions of Temperament Survey (DOTS) and Achenbach's Child Behavior Check List (CBCL). With the assistance of another researcher, children completed instruments designed to measure their self esteem, social support networks, perceptions of responsibility, predictability and control of domestic violence, social problem solving skills, and perceptions of their peers and parents. Teachers provided information concerning

the children's academic performance and behavior using the Teacher CBCL and the Harter teacher measures. Peers provided information about their classmates by participating in the Classroom Play, a sociometric technique. Three years after the initial phase of data collection, family caseworkers completed questionnaires focusing on changes in family circumstances and current parent-child relationships. Homeroom teachers later provided information about the children's classroom behavior, academic performance and peer relationships using the Teacher CBCL and the Taxonomy of Problematic Situations (TOPS). Mental health professionals and school personnel provided information about children's psychological functioning, truancy, special educational needs, physical health, delinquency, and performance on standardized tests. In FY 94 and FY 95, the children, as well as their mothers and fathers, were reinterviewed. Parents were asked to describe their children's behavioral problems using the CBCL. Children completed the Child Assessment Survey (CAS) to permit exploration of any psychopathology, the YSR to describe behavior problems, the Children's Depression Index (CDI) to measure depression, and a variety of other measures designed to assess their level of functioning in diverse developmental domains. The final interviews will take place early in FY96.

Children's testimony: In the second study, a large sample of audio- and videotaped victim/witness statements elicited by professionals are being collected from several different sites in the United States and Israel. The richness of these statements is systematically evaluated by research assistants using the Criterion Based Content Analysis (CBCA) scoring system and compared with independent ratings of forensic evidence about the case, including medical evidence, material evidence, and witness or suspect statements. The results of the statement analyses are being compared with case facts to assess the validity of the children's accounts and the accuracy of the procedure. The quality of interview is also being evaluated by examining the association between the types of questions posed by interviewers and the amount and quality of information provided by the children. In this research, coders focus on five types of interviewer utterance--open-ended or invitational, facilitative, directive, leading, and suggestive--as well as the length (number of words) and richness (number of details) of the children's responses. This information is also being used to explore how the quality of interview, indexed by the strategies employed to elicit information, is related to CBCA scores. Based on the results of these studies, SSED staff developed and are now field-testing interview protocols designed to increase the amount and quality of information provided by young victims of alleged abuse. In one other current study, we are examining the effects on interview style and children's testimony (as assessed by the richness and length of their accounts) of the use of anatomically detailed dolls. In another, we are systematically evaluating the length and richness of interviews in relation to the length of time that has passed since the children were allegedly abused.

Major Findings:

Effects of Domestic Violence: Overall, the results demonstrate that domestic violence has effects on child development that vary in nature depending on the type of violence and the source of information about the child's adjustment. In spite of the fact that the behavior problem scales were standardized separately for boys and girls, the girls in this sample were found to be more adversely affected than their male counterparts. No findings are yet available from the followup of children who have experienced domestic violence.

Children's Testimony: In several samples, we have confirmed the prediction that open-ended invitations yielded significantly longer and more detailed responses than directive, leading, or

suggestive utterances regardless of age and culture (Israel, United States). Younger children provide briefer and less detailed responses, however. Detailed psycholinguistic analyses of 45 interviews of 4- to 12-year-old children by police investigators in the United States revealed that the superiority of invitations was greater when the children reported experiencing three or more, rather than only one, incidents of abuse. Invitations were rarely used, however, and the investigators often failed to elicit more information from children who reported multiple incidents of abuse than from children who reported only one incident. In a related study designed to evaluate the relative effectiveness of two techniques for motivating young witnesses (M = 8.5 years; range, 5 to 12 years) to provide detailed accounts of alleged experiences of sexual abuse, investigators used either of two interview protocols. In half of the interviews, 11 investigators who were naive with respect to the experimental hypotheses used a script containing many open-ended utterances to establish rapport, whereas in the other interviews the same investigators used a script involving many direct questions. Both introductory scripts took about 7 minutes to complete and both included the identical open-ended statement to initiate the substantive phase of the interview. Children who had been trained in the open-ended condition provided 2 1/2 times as many details and words in response to the first substantive utterance as did children in the direct introduction condition. Children in the open-ended condition continued to respond more informatively to open-ended utterances in the later (unscripted) portion of the interview. Seventy-five percent of the children mentioned the core details of the incident in their responses to the first substantive utterance and a further 20% mentioned core details more vaguely.

Significance for Biomedical Research and the Program of the Institute:

Domestic violence is believed to have deleterious effects on children's development. Our initial study has been unique in its methodological rigor and in the scope of information collected. It was the first study in which four different informants--mothers, fathers, teachers, and children--reported on the incidence of the same set of behavior problems using the same measures. The major discrepancies found between reporters underscore the importance in both clinical and research contexts of obtaining information from multiple sources concerning children's adjustment. The findings also suggest that although abused children describe abusive parents more negatively than nonabusive parents, they also describe positive aspects of their relationships with their abusive parents. This information needs to be considered in clinical contexts, such as when child placement decisions are made.

Research on children's testimony is increasingly necessitated by the rapidly increasing numbers of allegations and the lack of reliable and accepted techniques for evaluating child allegations, especially those made by very young children. Research focusing on the impact of interviewer style on quality and quantity of information provided by children is urgently needed in the field, because such research permits the development of improved interview techniques. In addition, research on this topic promises to advance our understanding of children's memory for everyday events, supplementing a literature based on memory for contrived events.

Proposed Course of Project:

Domestic Violence:: We are currently completing re-interviews of the children and parents participating in our longitudinal study. These data, along with the data collected in 1993 and 1994 from the teachers and mental health professionals, have been entered into our data base. Data analyses will begin in FY96.

Children's Testimony: Evaluation of the CBCA system will be completed early in FY 96. Thereafter we will revise our coding system and expand the study in order to examine in more detail how the application of the CBCA system might differ depending on the child's age, the type of allegation, and the length of delay between the abusive incident and the child's statement. The effects of such factors on the quality of investigative interviews will also be explored. We will also continue field experiments designed to determine how children can best be "trained" to provide richer accounts of their experiences, and whether aids such as anatomically detailed dolls improve or impede children's accounts.

Publications:

Horowitz, SW, Lamb, ME, Esplin, PW, Boychuk, TD, Reiter-Lavery, L, Krispin, O. Establishing ground truth in studies of child sexual abuse. Expert Evidence, in press

Lamb ME. (Rapporteur) The investigation of child sexual abuse: An interdisciplinary consensus statement. Journal of Child Sexual Abuse, 1994; 3: 93-106; Child Abuse and Neglect, 1994; 18: 1021-1028

Lamb, ME, Hershkowitz, I, Sternberg, KJ, Esplin, PW, Hovav, M, Manor, T, Yudilevitch, L. Effects of investigative utterance types on Israeli children's responses. Internat J Behav Develop, in press

Lamb, ME, Sternberg, KJ, Esplin, PW. Making children into competent witnesses: Reactions to the amicus brief in re Michaels. Psychol, Pub Policy, and Law, in press

Sternberg, KJ, Lamb, ME, Dawud-Noursi, S. Does domestic violence produce behavior problems in children? In SS Luthar, JA Burack, D Cicchetti, JT Weisz (eds.), Developmental psychopathology: perspectives on risk and disorder (essays in honor of Edward F. Zigler). Hillsdale, NJ: Erlbaum, in press

Sternberg KJ, Lamb ME, Greenbaum C, Dawud S, Cortes RM, Lorey F. Effects of domestic violence on children's perceptions of their perpetrating and non-perpetating parents. Internat J Behav Devel, 1994; 17: 779-795

Sternberg, KJ, Lamb, ME, Hershkowitz, I. Child victims and witnesses in Israel. In BL Bottoms, GS Goodman (eds.), International perspectives on child witnesses. Thousand Oaks, CA: Sage Publications (in press)

Sternberg, KJ, Lamb, ME, & Hershkowitz, I. Child victims and witnesses in Israel. Criminal Justice and Behav, in press

Sternberg, KJ, Noursi, SD. Effects of domestic violence on children's behavior problems: Multiple perspectives. In R Tessier, GM Tarabulsy (eds.), Child and Family: Contexts for development. Quebec City, Quebec, QU: Les presses de l'Universite Laval (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01116-08 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Social Development Across the Lifespan in Diverse Cultures and Ecologies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	M. E. Lamb	Head, SSED	LCE, NICHD
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	S. Noursi	Pre-IRTA Fellow	LCE, NICHD

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LAB/BRANCH

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SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The work on this project involves a number of studies in a variety of cultures. The overall objective is to explore the ways in which developmental environments can be described by variations in physical and social ecologies, especially in terms of parental beliefs, values, and practices, and to assess how differences in these domains affect children's development. In one study, Palestinian mothers, grandmothers, and great-grandmothers are being interviewed about their perceptions, values, expectations, responsibilities, and practices. The goal is to explore transgenerational changes in childrearing practices and beliefs in a culture exposed to tremendous exogenous influence, especially over the last half century. In a second study, researchers are attempting to assess specific maternal and child attributions about one another in order to identify the extent to which attributions or expectations shape the way that parents and children interact, with particular focus on the development of aggressive behavior. Mothers and sons interact more aggressively when they had negative attributions of one another and recent analyses suggest that maternal and filial attributions affect future aggressive behavior. In the third study, gender differences in the self-perceptions of young adolescents are being assessed so that staff can explore the antecedents and correlates of different styles of self-perception.

Project Description:

Objectives:

Human development is the result of a complex interplay of multiple influences. Just as there is much variability in human physiognomy across races and ecologies, so too is there remarkable variety in childrearing patterns across cultures and ecologies. An ecocultural research perspective on child care is likely to provide unique insights for understanding how diverse cultures cope with the childcare challenges they face and how they actually design, provide, or ensure the provision of childcare. The short-term objectives of the project are to identify and explore the various properties that characterize different rearing ecologies and to describe some of the intrapsychic processes that may be influenced by environmental variations. The project's long-term goal is to compare and contrast developmental processes across developmental niches in order to identify universal or transcultural principles that underlie the development of human beings. To address these broad goals, studies have been undertaken to describe childrearing contexts, the dynamics of intrapsychic processes, and the processes whereby parental beliefs affect parental behavior and thus children's development.

In one study, initiated in 1990, procedures were developed to assess mothers' and children's attributions about one anothers' intent. Individual differences in these attributions were expected to predict differences in the coerciveness of their interactions. Cross-sectional and longitudinal studies were designed to explore these associations developmentally.

The most recently initiated studies are concerned with gender differences. In one, the female members of three-generational Moslem families in Palestine are being interviewed to explore changing conceptions of gender differences and their implications for socialization in a rapidly changing sociocultural context. In the other, procedures were developed to study gender-linked aspects of self-identity as well as the family and cultural antecedents of difficulties with self-identity among young adolescents. The research is designed to address Gilligan's theory that there are profound differences in male and female senses of self and maturity, as well as gender differences in the "fear of success".

Methods Employed:

This project comprises several studies in diverse settings that possess distinctive ecocultural features. During FY95, effort was expended on three studies: One concerned with the effects of maternal and filial beliefs about one another on the quality of their interactions, and the others with gender-related differences in parental beliefs, socialization practices, and self-perceptions.

In the first study, collaborators in North Carolina questioned mothers and their 7-9 year old sons concerning their expectations, beliefs, and attributions about one another and then observed the mother-child dyads in semistructured interactions designed to elicit cooperative or competitive strategies. In addition, teachers described the children's classroom performance and their interaction with peers. Using both cross-sectional and longitudinal data analyses, the goal is to examine the extent to which negative attributions and expectation of hostile behavior contribute to the development of aggressive behavior on the part of young children from diverse socioeconomic

backgrounds. Data reduction is now complete and analyses are in progress. The first phase involved 104 mothers and sons; the second some 240 mothers and sons, each interviewed and observed twice, one year apart.

The second study involves open-ended and structured interviews with the great-grandmothers, grandmothers, and mothers in three-generational Moslem families in Abu-Ghosh, an Arab village near Jerusalem. The interview format was finalized after extensive pilot-testing in other Arab villages. Staff interviewed informants using a protocol developed for this study; interviews were tape recorded for later coding and analysis.

In the third study, more than 100 12- to 15-year-old adolescents of both genders a) responded to two story stems concerning conflicted accounts of success, b) completed a questionnaire concerned with guilt and reparation, and c) described themselves in a variety of roles and contexts ("as I usually am", "as I would like to be", "as I am with my mother", etc.) using 50 self-descriptive adjectives chosen from a list presented to them. The data were computer coded for data analysis.

Major findings:

In the first study, both maternal and child attributions, as revealed in interviews about one another, were significantly related to the coerciveness of their interactions, as evident in two game-like tasks (Trouble, Etch-A-Sketch). The most aggressive dyads were those in which both mothers and sons perceived hostile intent in the other. Mothers' (but not sons') attributions were also related to teachers' reports of the children's aggression in the classroom. Boys who reported many stressful events in their lives behaved coercively with their mothers and were viewed by their teachers as more aggressive and less socially competent with peers. The relation between stressful life events and the boys' reported aggression with their peers was mediated, in part, by the boys' coerciveness with their mothers. The association between boys' coerciveness with their mothers and social acceptance by peers appeared to be mediated by the aggressiveness of the reported interactions with peers. Longitudinal analysis now in progress suggest that attributions appear to have a causal influence on aggressive behavior.

Three generations of Palestinian mothers had surprisingly similar views of gender differentiated practices and behavior. The social ecologies and parental expectations regarding marriage were quite different for boys and girls, as expected, although the expected relaxation over successive generations was not found. Mothers in all three cohorts maintained traditional views and practices.

The other study has yet to yield results.

Significance for Biomedical Research and the Program of the Institute:

The significance of the project lies in the focus on disparate cultures in an attempt to elucidate normative developmental processes. By obtaining data about development in diverse Western and non-Western societies, the results extend our knowledge of development and underscore the need to understand the broader socio-cultural contexts in which development takes place. This will enable us to characterize the relationship between environments and developmental processes, which is especially important at this time of increasing complaints about the monocultural nature of

developmental psychology as well as concern about the limited universality of developmental norms and principles. Similar concerns apply to the historical preoccupation with the development of males.

Future Goals:

Longitudinal data gathered on parental and child beliefs, attributions, and behavior in Greensboro, NC will be used to explore patterns of mutual influence and change over time, as well as the relationships between attributions and later peer acceptance. In addition, plans are being made to follow these children, and a comparable group of girls, as they move into adolescence. Analyses of gender-related differences in self-identity will be completed in FY 96, as will the preliminary review of the Arab women's attitudes and beliefs. Gender-related differences in self-identity will next be studied in other sociocultural contexts.

Publications:

Hwang CP, Lamb ME, Sigel I (eds.), Images of childhood. Hillsdale, NJ: Lawrence Erlbaum Associates, in press

Hwang CP, Lamb ME, Sigel I. Images of childhood: Some concluding thoughts. In CP Hwang, ME Lamb, I Sigel (eds.), Images of childhood. Hillsdale, NJ: Lawrence Erlbaum Associates, in press

Lamb ME. Review of John Snarey's "How fathers care for the next generation: A four decade study". Hum Develop, 1994; 37: 385-387

Lamb ME. "Response to commentary on early contact, bonding, and the development of mother-infant relationships. J Devel Behav Pediatrics, 1994; 15: 384-385

Lamb ME. What is selected in group selection? Behav and Brain Sciences, in press.

Lamb, ME. De invloed van de vader op de ontwikkeling van het kind. [The influence of the father on the development of the child]. Familia, 1994; 1: 53-64 [Dutch]

Lamb, ME. L'influence du pere sur le developpement de l'enfant. [Paternal influences on child development]. Enfance, in press [French]

Lamb, ME. (ed.) The role of the father in child development (Third edition). New York: Wiley, in press

Lamb, ME. The role of the father in child development: An introduction and overview. In ME Lamb (ed.), The role of the father in child development (Third edition). New York: Wiley, in press

Lamb, ME. The development of father-infant relationships. In M.E. Lamb (ed.), The role of the father in child development (Third edition). New York: Wiley, in press

Lamb, ME, Billings, LL. Fathers of children with special needs. In ME Lamb (ed.), The role of the father in child development (Third edition). New York: Wiley, in press

Lamb ME, Hwang CP. Images of childhood: An introduction. In CP Hwang, ME Lamb, I Sigel (eds.), Images of childhood. Hillsdale, NJ: Lawrence Erlbaum Associates, in press

Nsamenang, AB, Lamb, ME. The force of beliefs: How the parental values of the Nso of Northwest Cameroon shape children's progress toward adult models. J Applied Develop Psychol, in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01117-08 LCE

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Hospitalization Experience: Children's Coping with the Stress of Surgery

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. H. Bornstein	Head	LCE, NICHD
	J. L. Genevro	IRTA Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Child and Family Research

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research examines contributions of developmental and individual-differences factors to children's understanding of routine medical care and of strategies for coping with distressing medical experiences. The research integrates the adult stress and coping literature, research on changes in children's cognitive capabilities as they mature, literature pertaining to preparing children for hospitalization, and recent theoretical work regarding the effects of routine pediatric health problems and treatment on child development.

Project Description:

Objectives:

The primary goal of this research is to determine how developmental processes (e.g., changes in cognitive and emotion-management capabilities) and individual differences (e.g., gender) influence children's understanding, emotions, and behavior in routine and aversive medical care situations. In this research, social-cognitive developmental theory is applied to understanding children's capabilities in the face of stresses related to their physical well-being. Specific research questions include how cognitive developmental levels influence (1) children's comprehension of routine pediatric health care experiences and (2) their knowledge of and preferences for strategies for coping in stressful medical situations. The project also examines complex relations among coping and variables such as children's problem-solving ability, gender, anxiety, and parental anxiety that may moderate or mediate relations between age and coping knowledge and behavior.

Methods Employed:

Three major studies have been conducted, the first on children's opinions; the second on children's reactions before hospitalization, on the threshold of surgery, and after hospitalization; and the third on cognitive and affective dimensions of young children's mental representations of routine medical care. Two additional studies are in process: The first, conducted with collaborators at the Yale University School of Medicine, is in its initial stages; the second is a study of health and development in participants in the longitudinal study of maternal-child interaction ongoing in the CFRS.

The first major study, on children's opinions, involved school-aged children not experiencing or facing any unusually stressful events. In this study, for which data collection and analyses have been completed, 75 children between 5 and 12 years who were not anticipating hospitalization or surgery were interviewed concerning their knowledge of coping. Coding schemes were developed for categorizing the coping strategies proposed by subjects. Children's responses were grouped into categories based on coping techniques discussed in the adult coping literature, on a continuum from approach to avoidance techniques, with emotion-manipulation techniques lying equally between these two poles. Avoidance tactics, the main focus of interest for the management of uncontrollable stress (such as hospitalization), were further divided into partial (cognitive and behavioral distraction) and complete (escape and denial) types. Cognitive distraction includes fantasy and thinking about something else; behavioral distraction includes playing and watching television; escape is leaving the situation or going to sleep; and denial involves suppressing and forgetting. These coding schemes were later used to analyze children's responses to the coping interviews in the hospitalization study. Identical coding schemes were used for both investigations to facilitate the comparison between children's coping assessed in neutral settings and coping assessed in a real-life stressful situation.

Participants in the second major study, which focused on children's reactions to

hospitalization, were 62 children between the ages of 5 and 13 years hospitalized for elective surgery in a large, inner-city hospital and their parents. This study is unique in that children and parents were observed and interviewed at multiple time periods before, during, and after hospitalization: (1) one week before a pre-admission program, (2) the morning of the pre-admission program, (3) the day of admission, (4) the morning of surgery (before leaving the ward to go to the operating room), (5) waiting outside the operating room, (6) entering the operating room, (7) the morning after surgery, (8) one-week after surgery, and (9) one month after surgery. Self-report, parent-report, and observational measures were used to assess aspects of children's knowledge of coping strategies, information seeking, expectations, level of anxiety, and behavioral responses to the experience.

The third major study, which investigated the nature of children's representations of routine medical care, included 70 children and their mothers. Children were interviewed at 48 months of age. Transcripts of children's knowledge of routine pediatric care events were coded for cognitive structure and content. Transcripts were also coded for emotion content. Measures completed by each mother included assessments of her child's health status and the mother's preferred methods of treating her child's physical symptoms. The latter included ratings of the degree of concern/worry each mother felt about her child's health, relative to her perceptions of other mothers of children the same age, and her perceptions of her child's general health.

The study undertaken in collaboration with colleagues at the Yale University School of Medicine is targeted toward the investigation of preoperative anxiety in children and parents. Subjects are children between the ages of 5 and 12 years who are admitted for outpatient surgery. Measures include the coping interview used with children in studies 1 and 2 described above, a coping questionnaire for parents based on a modification of the coping interview, measures of child and parent anxiety, observations of child behaviors during anesthesia induction, and measures of recovery from surgery. Analyses will focus on relations among age, coping strategy knowledge, and parental and child anxiety as predictors of children's behaviors and recovery.

Data collection for the study of children's physical health and development is complete. Medical records of approximately 160 48-month-olds have been coded (information derived includes, for example, total number of visits to pediatricians, emergency rooms; well visits; sick visits; diagnoses; referrals for developmental evaluation, surgeries, etc.; number of illness episodes; duration of illness episodes). Questionnaire data were also obtained from mothers regarding their perceptions of their children's susceptibility to illness and general health status, and their own propensity to medicate their children, seek pediatric care for specific illnesses and conditions, and a number of other factors related to health attitudes and behaviors. Analyses of these data will include investigations of a number of current questions in development and health, such as relations between recurrent otitis media and language and behavioral development, and child and maternal predictors of pediatric health care utilization.

Major Findings:

The first three studies are complete. Coding schemes for several open-ended measures in the study have been developed, and protocols for those measures have been coded. Data analyses focusing on age-related differences in children's ratings of the value and efficacy of specific coping strategies in relation to: (1) coping by a child in a hypothetical situation and (2) their own coping efforts during hospitalization have been completed; the report of these analyses is currently under review for publication. Additional analyses currently underway address questions about relations between children's anxiety, specific medical fears, coping knowledge, and coping behaviors during a stressful pre-hospitalization procedure (phlebotomy).

Although information about children's knowledge and use of distraction strategies is slowly accumulating, little is known about children's perceptions of the usefulness of distraction strategies in helping them cope with different types of distress. Our research on children's coping has focused on children's knowledge of strategies for coping with potentially distressing experiences in which avoidance coping strategies may be particularly useful. As a result of this research, we have learned about the coping strategies school-aged children are able to articulate in response to questions about coping in hypothetically stressful situations. Less is known, however, about children's estimations of the value (how good a strategy is for dealing with a distressing situation) and efficacy (whether the strategy will change feelings of fear and/or distress) of specific strategies.

We have therefore investigated children's perceptions of the value and efficacy of strategies for coping with uncontrollable stressful situations, given findings in the adult literature that certain types of avoidance strategies have been characterized as more or less effective. Because conclusions about which strategies work best for adults have often provided the foundation for programs designed to help children cope with stressful medical experience, it is important to determine the ways in which children's perceptions differ from or model findings from research with adults. In addition, the possibility that the experiences of being hospitalized and having surgery affect children's perceptions of the value and efficacy of specific strategies was investigated. Our work in this area was guided by these questions: (1) How valuable do children consider different strategies for coping with the stresses associated with undergoing medical treatment? (2) Which strategies do children think are likely to change feelings of fear about undergoing medical treatment? (3) Do children's perceptions of the value and efficacy of coping strategies differ as a function of age? (4) Does age predict children's reports of strategy use? (5) Do children's estimations of strategy value and efficacy predict which strategies they report having used while hospitalized? (6) Do children's perceptions of the value and/or efficacy of specific strategies change as the result of being hospitalized? (7) Do children's perceptions of strategy value and efficacy change based on strategy use while they were hospitalized? In addition to proposing these general questions, we specifically hypothesized, based on related research on children's coping and problem-solving abilities (e.g., Altshuler, Genevro, Ruble, & Bornstein, 1995), that age would relate positively to children's perceptions of the value and efficacy of cognitively mediated avoidance strategies (e.g., cognitive distraction). Because being hospitalized and having surgery are experiences over which children typically can exert little or no control, we also hypothesized that estimations of the value and efficacy of specific emotion-focused

strategies (behavioral distraction and cognitive distraction) would increase from pre- to posthospitalization. Finally, based on previous related research on uncontrollable stressful situations, we anticipated that children would most frequently report using behavioral distraction strategies to cope with feelings of fear while hospitalized.

Analyses indicated that compliance and the availability of social support may be slightly more important coping tactics for children than for most adults, but also that both behavioral and cognitive distraction strategies play highly valued roles in children's repertoires for coping with distressing medical experiences. Results indicating that older children were more likely to consider cognitive distraction a useful strategy were consistent with results of analyses examining children's knowledge of coping strategies. In addition, children who believed that behavioral distraction strategies were likely to change their feelings were more likely to report having used those strategies while hospitalized.

To examine the genesis of children's knowledge and understanding of strategies for coping with distressing medical experiences, we have also begun to examine the role played by children's representations of their medical experiences as a means by which cognition of and emotional responses to distressing events may be linked. We have also examined relations among parental health perceptions, anxiety, and children's mental representations of medical care to investigate the premise that children's mental representations provide an avenue by which parental anxiety may influence children's knowledge and enactment of specific coping strategies. We propose that children's mental representations of routine medical care include not only the events experienced but children's affective responses. These elaborated mental representations, therefore, may serve as a foundation for the development of children's abilities to cope adaptively with distressing medical treatment.

Results from the study of 48-month-old children's mental representations indicated that most subjects' representations included differentiated details about routine pediatric care, including specific events experienced and associated affective responses. We found, as hypothesized, that mothers' concerns about their children's health were related to the content and structure of children's representations of the event of going to the doctor. This finding supports further investigation of the premise that children's mental representations serve as a mechanism by which parental attitudes and behaviors, and children's early experiences with medical care, influence the development of coping knowledge and behaviors. Results of the study also suggest that gender differences in children's representations of routine pediatric care, and in affective responses to that care, should be examined in future research.

One additional result of our work in the areas of children's development and health was the NICHD-sponsored workshop entitled Child Development and Behavioral Pediatrics: Toward Understanding Children and Health. This multidiscplinary conference, which we organized, was held in March, 1995 immediately prior to the biennial meeting of the Society for Research in Child Development. The workshop resulted in an edited volume that will be published by Lawrence Erlbaum Associates in 1996.

A integrative review of research relevant to developmental issues in children's coping with aversive medical experience, which incorporates recent research on cognitive development and emotion regulation is underway. Topics covered in this review include influences of individual differences factors (such as temperament) on coping, and children's use information to manage distressing emotions, because research on children and adults suggests that individuals differ in the extent to which they approach or avoid information relevant to threatening or challenging situations. The review also includes a discussion of developmental changes that may affect children's ability to manage their own distress and behaviors during a crisis. Because hospitalization is often particularly stressful for young children, it is important to ascertain what effects negative emotional arousal may have on children's abilities to access and enact coping strategies when confronted with stress. The roles of parents and other adults (such as medical personnel) in the coping process are considered.

Significance of Biomedical Research and the Program of the Institute:

As previous findings indicate that young children are often unable to manage pain or fear on their own, it is important to determine whether parents and hospital staff can effectively bolster children's efforts to manage their emotions in distressing and painful medical treatment situations. In order to ascertain which coping tactics are favored by children in specific situations, which tactics are most effectively used by which children under what circumstances, and who children consider to be effective "coping partners," new interview measures of children's coping knowledge and expectations are being developed based on the findings of these studies. The identification of the effects of variables, such as problem solving, gender, and anxiety, on coping and on relations between age and coping, also may make it possible to more successfully individualize programs preparing children for stressful medical procedures. The findings, both extant and emerging, of this project hold the promise of contributing in important ways to refining theories of cognitive development and coping. The effects of development on coping -- and the influences of coping on development -- are important pieces of the puzzle of relations between challenge and adaptation. The convergence in this project of cognitive-developmental theory with issues in the practice of behavioral pediatrics expands the realm of possible contributions of this research program. These potential contributions include measures to be used in other NICHD research, enhancements of the treatment of hospitalized children based on developmental theory, and revision of developmental theory based on the study of children in not uncommon stressful situations.

Proposed Course of Project:

Data collection for four studies contributing to this project is complete, and data collection in the collaborative project at the Yale University Medical School is underway. Results of analyses conducted to date suggest that relations between age and coping are affected in complex ways by individual differences factors such as gender and level of cognitive development. Based on these results, new interview measures of children's

coping knowledge and expectations have been developed. Analyses, both continuing and planned, are designed to: (1) explore relations among anxiety (parental and child), coping knowledge, and coping behaviors; (2) further investigate the role played by children's mental representations of medical care in the development of coping knowledge, coping behaviors, and adaption to distressing medical experiences; and (3) investigate relations between physical health status and developmental processes and outcomes.

Publications and Presentations:

Altshuler, JL, Genevro, JL, Ruble, DN, & Bornstein, MH. Children's knowledge and use of coping strategies during hospitalization for elective surgery. J Appl Dev Psychol 1995;16:53-76.

Bornstein, MH, & Genevro, JL eds. Child development and behavioral pediatrics: Toward understanding children and health. Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Bornstein, MH, & Genevro, JL. Child development and behavioral pediatrics. In: Bornstein MH, Genevro JL, eds. Child development and behavioral pediatrics: Toward understanding children and health. Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Genevro, JL, Andreassen, CJ, Baggeroer, CE, & Ainbinder, AM. What happens when you go to the doctor: Preschoolers' knowledge of routine pediatric care. Ped Res 1994;35:21A.

Genevro, JL, Andreassen, CJ, & Bornstein, MH. Children's responses to medical procedures: Developmental and experiential contributions to coping knowledge and coping preferences. In: Bornstein MH, Genevro, JL, eds. Child development and behavioral pediatrics: Toward understanding children and health. Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HD 01119-08 LCE

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Specificity of Mother-Infant Interaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD
J. Suwalsky	Research Psychologist	LCE, NICHD
K. Painter	Research Psychologist	LCE, NICHD
M. Fivel	Research Psychologist	LCE, NICHD
C. Andreassen	IRTA Fellow	LCE, NICHD
C. Rahn	Research Psychologist	LCE, NICHD
M. Haynes	Statistician	LCE, NICHD

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Child and Family Research Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	1.8	2.8

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates endogenous and exogenous factors that contribute to the development of cognitive and social competencies in children during the first four years of life. Before children are old enough to enter formal social learning situations, nearly all of their experiences stem directly from interactions they have with their primary caregivers. The present study focuses on the extent to which several maternal characteristics (age, parenthood status, SES, parity, employment status as well as type of substitute care experienced during mother's employment) influence observed relations between caregiving on the one hand and child developing cognitive and social competencies on the other. In this longitudinal study, subjects are visited when they are 5, 20, and 48 months of age. At 5 months, mother-infant interaction is observed. At 20 months, measures of toddler functioning (play competence, language development, and social adaptation) and maternal behavior (play and I.Q.) are obtained. At 48 months, the follow-up examines diverse children's abilities (representational competence, problem solving, reasoning skills, mathematical ability, language use and discourse, and selected aspects of generally adaptive behavior).

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: This longitudinal project has several aims. The first is to investigate the influences of caregiver behaviors and beliefs on cognitive and social development in infancy and early childhood. Two classes of caregiver behavior -- social and didactic modes of interaction -- are linked to specific concurrent perceptual-cognitive and exploratory competencies of babies in their first six months of life. In this investigation samples of mothers and infants varying on characteristics of the caregivers that might differentially influence the behaviors and beliefs of interest are being observed. The present study set addresses questions of whether and to what extent maternal and infant behaviors, and relations between them, are influenced by the following factors: maternal age (adolescent, younger, older), maternal employment status (homemaker, employed), type of substitute care experienced in connection with mothers' employment (one-on-one care in the baby's own home by a single adult, family daycare), parenthood status (mother by adoption or by birth), SES, and birth order of infant (first- vs. secondborn).

A second major component of the study is to examine aspects of social and cognitive functioning in the child in the second year of life, both in relation to early experiences and as a function of group membership. The literature contains evidence that aspects of maternal behavior in infancy influence the development of cognitive and social skills in young children. Relations between carefully operationalized aspects of maternal behavior in the first and second years and aspects of the emerging capacity for mental representation in the child (e.g., language acquisition and production, level of play competence) in the second year support the view that such influences are multidimensional and complex. In the present sample, infants seen at 5 months are revisited when they are 20 months old, thereby permitting examination of relations between maternal and infant behavior in early infancy and toddler functioning at the end of the second year of life.

The third phase of this longitudinal study involves assessing a broad range of abilities in the child at preschool age. Measures of the following are obtained: the child's I.Q., creativity, self-concept, play and exploratory competence, mathematical skills, symbolic representation, language and storytelling skills, drawing and singing abilities, social cognitive abilities, and cognitive problem-solving skills. An understanding of how maternal behavior influences the child's functioning in social and cognitive spheres, both concurrently and longitudinally, will contribute importantly to our understanding of more general issues in preschooler cognitive development, such as how children make sense of and represent their experiences, how children acquire and use cognitive strategies, and how children learn to plan future activities and to use metacognition, self-monitoring, and self-regulation to anticipate task requirements and to evaluate ongoing performance. Although the question of maternal influence on preschooler cognitive development is largely unexplored, work with younger children suggests some avenues of approach for accessing these processes.

Ancillary studies. In several supporting and converging investigations in the laboratory, major dependent variables that are being examined in the larger longitudinal project are being scrutinized further. In one, language performance and play sophistication have been investigated in a longitudinal study of approximately 40 children seen at 2, 3, and 4 years of age. First, language and play at 2 years of age have been examined under a systematically varied set of experimental conditions: with mother or stranger, in the home or laboratory. This methodological study was designed to help determine the best estimators of individual differences in child language and play status. Perhaps, for example, strangers "lower the gain" in children's verbal output. To what degree? How much, relative to a strange situation? In different representational modes (language and play) or just one (language or play)? The results of this study will allow further insight into how social and physical context affect the expression of language and play in the young child, and they are intended to inform a reasoned choice of instrument, condition, and metric for future studies designed to assess central issues at the intersection of young children's cognitive and social functioning. In this same study, stability of performance from 24 to 36 months for mother and child with respect to language and play will be assessed.

Further, at 48 months, measures of the child's "theory of mind" have been obtained. Recent research on early development has focused on the young child's understanding that individuals have internal and unobservable desires and beliefs that explain their observable behavior, or a "theory of mind." Antecedents of this knowledge will be evaluated in children's early mental representational ability as expressed in their symbolic play and language, as well as in parent-child social interaction. Individual differences in the theory of mind of children 4 years of age are also being explored. We hypothesize that children who were more advanced on measures that assess mental representational ability early in life (language and play) will be more sophisticated on this task.

Another study related conceptually to the main longitudinal design is organized to document the influence of the loss of a major communication channel (deafness) on mother-child interaction and on children's developing language and play performance during the second year of life in order to explore the role of children's early language learning environments on the development of symbolic activity. Deafness in a family has a profound impact on the child's language-learning environment, and this impact will be mediated by whether the parents or child or both are deaf. For a deaf child, hearing and deaf parents provide significantly different language-learning opportunities and, by the same token, the language-learning environments provided by deaf parents of hearing and deaf children also differ. Language variation among children at the start of the second year has been shown to be associated with variation in their play sophistication, suggesting that these twin emerging abilities might reflect a transition in a common underlying "representational competence." Deafness either of the child or in the family may also be associated with variability in play sophistication. Such variability, if found, might help elucidate our understanding of the underlying "representational competence" linking language and play. Data are being collected in a sample of 80 subjects, divided equally among four groups: hearing mothers with hearing toddlers; hearing mothers with deaf toddlers; deaf mothers with hearing toddlers; and deaf mothers with deaf toddlers.

Finally, a collaborative, longitudinal study of children exposed to cocaine prenatally and postnatally has been initiated. A focus on young children from cocaine-abusing families represents a significant area for health psychology. Some major cities in the United States report 50% of women receiving prenatal care at inner city hospital clinics have used cocaine regularly during their pregnancy and for several years before. Further, an increasing number of children born to cocaine-using women and/or growing up with substance abusing parents (Scherling, 1994): No one estimate is certain, but currently perhaps more than 350,000 infants are born each year in the United States to mothers who have used cocaine with or without other substances (such as alcohol, marijuana, or tobacco) throughout their pregnancy. These twin trends have, in turn, led to two expanding areas of research, the first being a focus on the effects of prenatal cocaine exposure on infants' and children's development, and the second on studies of the characteristics of women who abuse substances and the effects of that substance abuse on their ability to parent. Children from cocaine-abusing families have multiple unmet developmental and health care needs, and their parents often have great difficulty meeting those needs or accessing care for themselves. When we look beyond the stereotypes and popular opinions, the problem of childhood cocaine exposure is marked by multiple levels of interaction between pre- and postnatal factors and between biologic vulnerabilities and disordered environments. Investigators seeking to understand further the effects of cocaine exposure on children's psychological development face the dilemma that cocaine affects development through multiple pathways. Because cocaine is a teratologic agent with profound effects on the child's immediate parental and more global social environment, as well as the child's pre- and postnatal development, studies of the outcome of cocaine-exposed infants necessarily involve biologic-environment interaction models. For the child and parent, the multiple pathways of effect of cocaine exposure also represent risks for numbers of other problems, such as homelessness, involvement in violence and crime, poor school performance and early school drop-out, and multi-generational drug abuse, problems that trap both parent and child in poverty and despair. Our studies examine the pathways through which prenatal cocaine exposure and ongoing parental cocaine use can affect both child and family. Understanding the developmental and physical needs of children from substance abusing families along with the needs of their parents will inform the development of more integrated health care services for children from substance abusing families.

The protocol follows basic features of the main CFRS longitudinal project. The study focuses on identifiable short-term, neonatal manifestations of cocaine exposure and on long-term effects on the infant's regulation of attention, state regulatory capacities, and the development of language and symbolic play. Many reports now document the neurophysiological manifestations of cocaine exposure in the immediate neonatal period, but little information exists about neurobehavioral development in these infants after the first one to two months. The neuropharmacological effects of cocaine in animals and adults, as well as clinical reports describing attentional impairments exhibited by preschool children exposed to cocaine prenatally, suggest that infants so exposed may be at continuing and serious risk for central nervous system impairment and dysfunction. This study has important implications for understanding how a mother's use of cocaine during pregnancy and her continuing abuse of drugs postnatally may jeopardize her infant's developing attentional, information-processing, and self-regulatory capacities through specific effects on fetal brain development and infant behaviors. We expect that information from this longitudinal study will also be useful in designing service programs for abused infants.

Methods Employed:

In the main longitudinal study, eight groups of (approximately) 30 primiparous mothers and their 5-month-old infants have been recruited. With the exception of an adolescent sample, all mothers are Caucasian, married women with healthy, term infants. The groups are composed of approximately equal numbers of male and female infants. A group of teenage mothers comparable to the other groups in terms of race, parity, and health status of the infant has been recruited, but there is variation on other dimensions within this group: for example, most of the teenage mothers are unmarried. Appropriate referral sources are being used for recruitment (well-baby clinics, adoption agencies and support groups, public school programs for teenage parents, and advertisements in newspapers and other pertinent publications). Volunteers are screened by telephone by trained members of the research staff.

The design of the study at the infancy age-point provides for six sets of comparisons. In each, one major independent variable is the focus of investigation; other independent variables (parental education, socioeconomic class, marital status, family intactness, the baby's antenatal care, circumstances of labor and delivery, birth order, the baby's health since birth, and postnatal alternate caregiving arrangements) that might influence the dependent variables being assessed are accounted for. Study I examines three groups, varying in terms of maternal age: teenage mothers with mean age less than 20 years, younger mothers with mean age between 20 and 30 years, and older mothers with mean age greater than 30 years. Study II is comprised of two groups varying in terms of mothers' employment status: homemaker mothers and mothers employed outside of the home. Study III includes two groups of employed mothers, differing in terms of the type of substitute care experienced by the infant during the mothers' absence: infants who experience one-on-one care by a single caregiver in their own home and infants who experience care by a single adult in that adult's home with other children present. Study IV includes two groups varying on parenthood status: mothers by birth and mothers by adoption. The group of biological mothers between the ages of 20 and 30 years is common to Studies I, II, and IV. Study V utilizes families varying in socioeconomic status. Study VI involves comparing the firstborn and secondborn infants of the same mothers.

Each mother-infant dyad is visited in the home setting when the baby is approximately 5 months old. At that time, a three-part videotape record of the infant is made. (In the following description, the mother is referenced. For two groups in the study, the baby is also seen with his or her substitute caregiver. The procedure is the same, regardless of the identity of the adult.) For the first hour, the mother is asked to go about her normal routine, and the infant's typical experience when at home with mother is filmed. Second, the infant is filmed alone with seven favored toys for 10 minutes. Finally, a 3-minute film is made of the mother holding the baby on her lap and interacting en face in a natural fashion. All visits are scheduled for a time when mother and infant are typically at home together and the infant is awake and alert. Mothers are encouraged to behave in their usual manner and to disregard the observer's presence insofar as possible. Mothers are also asked to complete a brief set of questions providing information on

demographic characteristics of mother, father, infant, and substitute caregiver (for those caregivers being observed), the baby's experience with substitute care, as well as aspects of the baby's birth or adoption. The videotapes are being coded by trained personnel using a behavioral system originally developed in connection with previous work in this area by the Principal Investigator and revised, expanded, and computerized for the current project. Throughout the course of the study, 20% of the tapes are being independently coded by two observers for purposes of assessing interobserver reliability.

Each mother and toddler dyad is visited again at home when the child is 20 months old. During the single 2-hour visit, the following data are gathered: measures of the child's play while he or she is playing alone and mother-child interaction during a joint play session, two measures of the child's language development (one obtained from the mother and the second assessed independently by the experimenter), a measure of maternal intelligence, and updated information about mother's employment and concomitant substitute care for the child that have occurred since the baby was 5 months of age. At the completion of the visit, the following areas are assessed: mothers' perceptions of the parental role, mothers' knowledge of various aspects of infant development, and aspects of maternal personality. Finally, information about the child's level of functioning with respect to communication skills, daily living skills, social responsiveness, and motor development is obtained.

At the third data point in the longitudinal study, the child is seen at 48 months of age in the laboratory playroom for one 3-hour visit. Data of various kinds are gathered from the child, including The Wechsler Preschool and Primary Scale of Intelligence-Revised, a standardized measure of verbal and performance abilities related to intelligence in young children.

In the first ancillary study, an independent sample of 32 children (16 males, 16 females) was recruited. Selection criteria included: Normal pregnancy/delivery status, intact families who are native U.S. citizens and are English-only speakers. At 24 and 36 months of age, the following data were collected: Language and play (at each age, naturalistic samples of each child's language and play have been audio- and videorecorded in two sessions, one session in the home and one in the laboratory), other measures of language performance, structured play, maternal intelligence, maternal perceptions of child temperament, and measures of maternal knowledge of infant development and perception of parenting behavior. At 48 months the children were given the Wechsler Preschool and Primary Scale of Intelligence -- Revised. At 48 months, measures of each child's "theory of mind" were also obtained. The children were questioned about the actions of characters in stories. Six tasks were used: (1) a "false belief task" which assessed the child's understanding that individuals can have beliefs or thoughts that do not correspond to reality; (2) a "relative certainty of mental verbs task" which assessed the child's understanding of the relative certainty of mental verbs in relation to external reality (e.g., that the mental verb "know" expresses more certainty than the mental verb "think"), in the context of a hiding game with puppets; (3) a "pretense as mental representation task" which assessed the child's understanding that pretending necessarily involves a mental representation as opposed to involving only action; (4) a "pantomime comprehension task" which assessed the child's ability to comprehend actions with imagined objects; (5) an "early referential communication task" which assessed the child's understanding of the effects of ambiguous and informative messages on their own knowledge state within the context of a game; and (6) an "understanding of emotions task" which assessed the child's understanding of emotions and feeling states. Data collection is complete in this study.

In the study of deafness and children's mental abilities, subjects were recruited with the help of schools for the deaf in Delaware, Maryland, New Jersey, Pennsylvania, Virginia, and West Virginia, as well as through newspaper advertisements. Groups were matched for child's age, socioeconomic status, parity, and gender, with deafness being the only disability involved. Each dyad was visited for approximately 2 hours in the home by two researchers and, when necessary, a deaf interpreter. The following data were gathered: videotapes of two 10-minute naturalistic play sessions, one with the child playing alone with a standard set of toys and one with the child and mother playing with the toys; an experimenter-administered "scaffolding" task in which the experimenter elicits increasingly sophisticated levels of play from the child; two measures

of the child's language development (one obtained from the mother and the second assessed independently by the experimenter); a measure of maternal intelligence; and descriptive information about the family. At the completion of the visit, the following areas were assessed: mothers' perceptions of the parental role, mothers' knowledge of various aspects of infant development, aspects of maternal personality, and sources of maternal stress. Mothers were asked to provide information about the child's typical play behavior by completing a play inventory and an activity diary. Finally, information about the child's level of functioning with respect to communication skills, daily living skills, social responsiveness, and motor development were obtained. In this study, data collection is complete and data analysis has begun.

In the study of infants born to drug-abusing mothers, infants exposed to cocaine are compared to infants whose mothers are drug-free. (The project is being conducted in collaboration with an extensive treatment program for cocaine-dependent pregnant mothers in which 300 women will be enrolled over a three-year period.) The cocaine-dependent group is matched to a control group by maternal socioeconomic status and for infant sex, birth order, birthweight, and gestational age. Neonatal assessments of the infants focus on neurological organization. After hospital discharge, infants are seen at 3, 6, 12, 18, and 24 months for sequential assessments of the regulation of attentional states, the capacity to sustain exploration, state organization, adaptive behavior development, language and symbolic play development, and mental age. Additionally, because of potential contributions of maternal behavior to the infant outcomes selected, we examine both the mother's interactive style with her infant, particularly in terms of attention-directing activities and the intervening maternal variables likely to influence the mother's ability to respond to the infant (psychopathology, life stresses, anxiety and depression, and sense of parenting competence). Data collection is ongoing at this time. Coding of maternal and infant interactive behaviors from videotaped sequences is in progress.

Major Findings:

Longitudinal Study--5 Months.

A computer-based system for the continuous coding of behavioral records developed within our laboratory has been adapted in a number of ways for use with the various data bases. With respect to the 5-month data, codes have been grouped into 13 "modes" which assess infant behavior (level of alertness, focus of visual attention, type of tactile exploration, facial affect, and type of vocalization), maternal behavior (vocalization, social play, mediation of infant attention to the social and inanimate environment, responsiveness, encouragement of gross motor control, and level of involvement with the infant), and aspects of the infant's environment (behavioral context, number and variety of physical locations in which the infant spends time, stimulus characteristics of the visual and auditory environments including detailed characteristics of play materials). Each mode consists of 5 to 8 mutually exclusive and exhaustive behavior categories. Each 60-minute videotape of naturally-occurring mother-infant interaction is coded 13 times, once with each mode. Much of this coding has been completed at 5 months in the longitudinal study.

At the infancy age-point, substantive analyses have focused on an in-depth examination of the data for the adoptive sample. The literature clearly documents that adoptive status places a child at risk for difficulties in the social and cognitive spheres. At the same time, little research attention has been paid to the experiences of adopted children and their families outside of clinical settings. An emerging literature is focusing on characteristics and patterns of behavior in so-called "normal" adoptive families as well as the developmental course of the "healthy" adopted child. To date, however, no observational studies of early mother-infant interaction have been reported.

A group of families by birth has been selected from the larger study population and matched to the adoptive sample in terms of sex of baby, SES, maternal age, maternal education, maternal employment status, and maternal occupation. At 5 months, birth and adoptive infants were compared in the frequency and duration of visual attention to their mothers and to objects, smiling and negative facial expression,

distress and nondistress vocalization, and touching and mouthing objects. A multivariate test, controlling for mothers' age, infant birthweight, and length of time that infants were in the home, indicated differences between birth and adoptive infants in the frequencies of one or more behaviors considered simultaneously. Follow-up univariate tests suggested that birth and adoptive infants consistently differed on four measures: in interaction with their mothers, birth infants visually explored objects, touched objects, and mouthed objects more often than adoptive infants and showed less negative expression. There were, however, no differences between birth and adoptive infants in the durations of the eight behaviors. These data provide inconsistent support for clinical reports that adoptive children show behavioral and cognitive deficits in early interactions with their mothers.

For their part, mothers of 5-month-old birth and adoptive infants were compared in the frequency and duration of speech, vocal imitation, social play, positive evaluation, encouraging attention to self and to objects, feeding, grooming, and holding during interactions with their infants. A multivariate test, controlling for mothers' age, infant birthweight, and length of time that infants were in the home, indicated no differences in the frequencies or durations of any of these behaviors in mothers of birth and adoptive infants. In this nonclinical group, there is no evidence that birth and adoptive mothers interact differently with their infants. Future analyses will investigate differences between birth and adoptive groups in the covariation of infant and maternal behaviors and will explore group differences at 20 and 48 months.

Relevant to the organization and understanding of the 5-month findings, several theoretical treatments of infancy, infant development, and parenting infants were published.

Longitudinal Study--20 Months.

Three codes have been developed to characterize the behavior of the toddler and the mother in the play sessions videotaped during the 20-month home visit. One graduated code has been developed to assess the extent to which the play of child and mother involves nonsymbolic versus symbolic actions. Using these same categories, the mothers' demonstrations and solicitations of toddler play are also assessed. The videotaped play sessions are coded to generate continuous computerized records of child and maternal play. These coding schemes have also been applied to the videotapes of child, mother, and experimenter play obtained in connection with the methodological and deaf studies.

Using data from the 20 month visit, sources of individual variation in child language competence has been examined. Maternal sociodemographic characteristics, personological characteristics, and language, as well as child gender, social competence, and language competence were evaluated simultaneously in 120 children and their mothers. Zero-order relations were obtained between child language and child gender with girls scoring consistently higher than boys. Children's social competence was also correlated with their language ability. Mother's language, personality, and SES were found to have a direct influence on children's language. Maternal verbal IQ, however, did not relate to child verbal comprehension or expressive language. Structural equation modelling supported the following unique predictive relations: Child gender and social competence predicted child language, and mother language predicted child language comprehension. Maternal language was influenced by SES and knowledge about parenting as well as by child gender.

Analyses were also conducted on the sources of individual variation in child pretense (symbolic) play in 130 toddlers at 20 months as an expression of emerging mental representation. Three categories of child pretense play have been defined: solitary pretense play, pretense play with the mother that the child initiated, and pretense play with the mother that the mother initiated. Mothers' pretense play with the child was of two kinds: demonstrations of pretense play and solicitations of pretense play. In three separate structural equation models, a latent variable representing each kind of child pretense play was formed from multiple indicators (proportions and absolute amounts of symbolic play based on frequencies and durations) and was used as the endmost dependent variable: children with high scores on the latent variable did a lot of symbolic play, proportionately and absolutely. In each model, predictors of child pretense play were

family socioeconomic status, mothers' verbal intelligence, exploratory personality, parenting attitudes, locus of control in parenting, parenting knowledge, affectionate behavior, social play, and symbolic play, as well as children's gender and language competence. A small percent (10.3%) of the variance in solitary pretense child play was directly predicted by mothers' verbal intelligence and child gender. There were no other direct or indirect influences on solitary play. A moderate percent (34.1%) of the variance in child-initiated collaborative pretense play was influenced directly by mothers' pretense play and indirectly by mothers' physical affection and verbal intelligence and child gender. An even larger percent (45.5%) of mother-initiated collaborative pretense play was influenced directly by mothers' pretense play and by child language competence and indirectly by mothers' physical affection and verbal intelligence and child gender.

Another set of analyses examined factors in mothers and their 20-month-old children that lead to mothers' beliefs that they are competent and satisfied in their parenting role. The question was investigated in 110 mothers and their firstborn children. Mothers' self-perceptions of competence and satisfaction in parenting were assessed using two scales from the Self-Perceptions of the Parental Role (MacPhee, Benson, & Bullock, 1986). Potential maternal predictors included socioeconomic status, age, hours of work per week, verbal intelligence, knowledge of infant development, dissonance in expectations for parenting in three domains (social, didactic, and discipline), personality trait of anxious sociability, and attributions of parenting failure to internal causes. Child predictors were gender, language comprehension, and social adaptability. In a structural equation model with self-perceptions of parenting competence as the dependent variable, higher dissonance in parenting expectations, more anxious sociability, and age predicted a lessening sense of competence; verbal intelligence positively predicted perceptions of competence mediated through less anxious sociability. None of the child measures influenced mothers' beliefs in parenting competence. In a second structural equation model with parenting satisfaction as the dependent variable, higher dissonance in parenting expectations predicted less satisfaction; no other maternal or child measure predicted parenting satisfaction, directly or indirectly.

Methodological Study -- 20 Months.

The methodological study has two related goals: first, to assess language performance and play sophistication in the same children under different conditions and at different ages and, second, to determine thereby the best estimators of individual differences in language and play status. Analysis of the play data at 20 months is underway. Preliminary analysis of the play data has revealed that a child's symbolic play performance is influenced by social context (solitary play vs play with mother vs play with experimenter), but not by location (home vs lab). Further, symbolic play performance does not differ according to the sex of the child.

In this same methodological study, the association between children's language development and other aspects of thinking that require symbolic functioning has been examined. Language comprehension in children was measured at 24 and 36 months using the Reynell Language Scales and was found to be stable across these two ages. At 24 months children were asked to perform actions with imaginary objects. At 36 and 48 months their ability to comprehend actions with imagined objects was measured. These measures of symbolic gesture ability at 24, 36, and 48 months were positively related, but only the correlation between 36 and 48 month gesture comprehension reached significance. Significant concurrent associations were found between language and gesture comprehension at 24 and at 36 months. All four of the significant concurrent and longitudinal relations between language and gesture remained high after 48-month IQ was partialed out. This pattern of results points to an underlying common factor in language and gesture that is separate from intellectual capacity as measured by traditional IQ measures.

Longitudinal Study--48 Months.

Although data collection at 4 years of age is not yet complete, two sets of preliminary analyses have been carried out. Both studies, using subsets of the longitudinal sample, have explored aspects of maternal teaching styles as observed during joint bookreading and jigsaw puzzle completion, the stability of these

teaching styles, and their relation to select child outcome measures. The first study, based on event representation theory, investigated the longitudinal contributions of the maternal modelling of symbolic sequential pretense play (observed at 20 months) and the concurrent contributions of maternal style during joint picture-book reading at 48 months to children's mental representations of a routine event (having lunch) as assessed using a standard event interview format. An important finding was the contribution of maternal references to the child's past experiences (e.g., "How did we make chocolate milk last week?") to the sophistication of children's event representations. The second study investigated the stability of maternal styles as observed during joint mother-toddler play at 20-months and joint mother-child jigsaw puzzle completion at 48-months. Hierarchical regression analyses suggested that maternal styles of interacting that place greater emphasis on representational competence show stability over time: Mothers who engaged in higher proportions of symbolic play at 20 months tended at 48 months to engage in higher proportions of teaching that emphasized evaluation and monitoring of progress during children's ongoing problem solving.

Finally, a multivolume handbook was organized and published on diverse aspects of parenting. These include the biology and ecology of parenting, the status and social conditions of parenting, and applied and practical aspects of parenting.

Cocaine Study

Several theoretical treatments and empirical studies were completed in this reporting period. One chapter reviews accumulated data, summarizes the principal research themes, and concludes with an overview of the health and developmental needs of substance abusing women and their children. While the potential effects of prenatal cocaine exposure on developing brain is the central question guiding our studies of cocaine exposure in children, other factors including the general postnatal environment and parental characteristics may also exert both direct effects on developing brain as well as on the developmental and behavioral domains regarded as the markers of direct central nervous system effects. No one pathway of cocaine exposure on development appears to be primary, each contributes variance to the relation between prenatal exposure and the child's developmental status, and so adequate study of the prenatally cocaine-exposed child must involve studies of parenting and the child's general environment as well. Other theoretical treatments review the effects of cocaine on child development at different levels. They include a survey of the effects of cocaine on central nervous system functioning and on developing fetal brain; a summary of available data on developmental outcomes in children exposed prenatally to cocaine; how cocaine abuse affects parental functioning; and an overview of prominent pathways through which the effects of cocaine exposure on basic neurodevelopmental functions in children may be mediated.

One experimental observation was completed to determine the effects of prenatal cocaine exposure on 3-month infants' information-processing and developmental assessments. One hundred and eight infants--61 cocaine exposed and 47 controls--participated in an infant-control habituation and novelty responsiveness procedure and in a developmental assessment using the Bayley Scales of Infant Development both administered by experimenters blind to the drug exposure status of the infant. Compared to the non-drug exposed group, infants exposed prenatally to cocaine were significantly more likely to fail to start the habituation procedure and, for those who did, significantly more likely to react with irritability early in the procedure. The majority of infants in both groups reached the habituation criterion, and among those who did there were no significant differences between cocaine and non-cocaine-exposed infants in habituation or in recovery of attention to a novel stimulus. Infants who were cocaine-exposed showed comparatively depressed performance on the motor (PDI) but not the mental (MDI) scales of the Bayley. These results obtained for habituation and Bayley MDI controlling for both perinatal and sociodemographic factors. These differences in reactivity to novelty, but not in information-processing, between cocaine-exposed and non-cocaine-exposed infants, suggest that the effects of prenatal cocaine exposure may be on arousal and attention regulation rather than early cognitive processes.

A second study was designed to investigate relations between drug exposure, particularly cocaine, and

infants' regulation of arousal in response to novelty more precisely. Sixty-three infants--36 cocaine-exposed and 27 non-cocaine-exposed--participated at 3 months of age in a novel-repeat stimulus presentation procedure. Arousal was operationalized in terms of infant behavioral state, affective response, and attention to the stimulus. Infants were tested and infant behaviors were scored by experimenters blind to the drug exposure status of the infant. There were no differences between the two groups in baseline behavioral state or affective expression before the presentation of novel stimuli. Compared to the non-cocaine exposed group, infants exposed prenatally to cocaine and other drugs were more likely to exhibit a crying state and to display negative affect on novel stimulus presentations. There were no group differences in the amount of looking toward the stimulus. Both groups showed less crying and negative affect when stimuli were presented a second time, but decrements were consistently greater for the cocaine-exposed group. These results obtained when group differences controlled for sociodemographic and perinatal variables.

Significance for Biomedical Research and the Program of the Institute:

This research contributes to a basic understanding of factors that shape the normative development of cognitive and social competencies in the infant and young child. The data collected at each time period permit in-depth examination of contemporaneous influences on emerging capabilities, whereas the longitudinal aspects of the project provide an invaluable opportunity to trace the developmental course of those competencies. Method development has emerged as an important aspect of this study set: Central questions with respect to measurement are being addressed, and innovative computer-based tools for data coding and analysis are being implemented. A unique and important aspect of the longitudinal study is the emphasis on a broad range of outcome measures in the cognitive and social spheres at the preschool-age period. Because traditional psychometric measures of intelligence in the preschool period typically sample from a narrow range of mental capabilities, such measures are limited in terms of the information they provide about the possible impact of antecedent variables. They are also restricted in terms of the outcome variables they successfully predict. By focusing on the "whole child," this investigation strives toward a better understanding of the complex, multidimensional, interdependent nature of growth and development in the human child. Last, but very importantly, the laboratory's work is probing a number of issues which have been identified by the NIH as critical problems with respect to the overall health and well-being of American women and children; these include: parenting among adolescent mothers; the effects upon children of maternal employment and various alternative forms of substitute care; the effects of substance abuse on early parenting behavior and infant development; and developmental sequelae for children whose lives are touched by deafness.

Proposed Course of Project:

Complete data have been obtained for nearly all of the 5-month groups in the longitudinal sample. Similarly, most mother-toddler dyads have been revisited at 20 months. Approximately 75% of the children have been revisited at 48 months of age. A follow-up when the children are 9 years old is in the planning stages and will commence in September, 1996.

Publications:

Bornstein, MH. eds. Handbook of parenting.
Volume 1: Children and parenting.
Volume 2: Biology and ecology of parenting.
Volume 3: Status and social conditions of parenting.
Volume 4: Applied and practical parenting.
Hillsdale, NJ: Lawrence Erlbaum Associates, 1995.

Bornstein, MH. Infancy. In: Sternberg RJ, Ceci SJ, Horn J, Hunt E, Matarazzo JD, Scarr S eds. Encyclopedia of human intelligence, Vol. 1. New York: Macmillan, 1994;570-5.

Bornstein, MH. Parenting infants. In: Bornstein MH ed. Handbook of parenting, Vol. 1. Mahwah, NJ: Lawrence Erlbaum Associates, 1995;3-39.

Lamb, ME, Nash, A, Teti, DM, & Bornstein, MH. Infancy. In Lewis M ed. Child and adolescent psychiatry: A comprehensive textbook, 2e. Baltimore: Williams & Wilkins, 1995;

Mayes, LC, & Bornstein, MH. Attention regulation in infants born at risk: Prematurity and prenatal cocaine exposure. In: Burack J., Enns JT eds. Attention, development, and psychopathology. (pp. xx-xx). New York: Guilford Press, 1995.

Mayes, LC, & Bornstein, MH. Developmental dilemmas for cocaine-abusing parents and their children. In: Lewis M, Bendersky M eds. Mothers, babies, and cocaine: The role of toxins in development. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers, 1995; 251-72.

Mayes, LC, & Bornstein, MH. The context of development for young children from cocaine-abusing families. In: Kato P, Mann T eds. Health psychology of special populations. New York: Plenum Press, 1995;

Mayes, LC, & Bornstein, MH. The development of children exposed prenatally to cocaine. In: Luthar S, Burak J, Cicchetti D, Weisz J eds. Developmental psychopathology: Perspectives on risk and disorder. New York: Cambridge University Press, 1996;

Mayes, LC, Bornstein, MH, Chawarska, K, & Granger, RH. Information processing and developmental assessments in three-month-old infants exposed prenatally to cocaine. Pediatr 1995;95:539-45.

Mayes, LC, Bornstein, MH, Chawarska, K, Haynes, OM, & Granger, R. H. Impaired regulation of arousal in three-month-old infants exposed prenatally to cocaine and other drugs. Dev Psychopath 1996;

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01120-08 LCE

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Parenting and Infant Activity: A Multiculture Perspective

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD
O. M. Haynes	Statistician	LCE, NICHD
C. Rahn	Research Psychologist	LCE, NICHD

COOPERATING UNITS *(if any)*
ARGENTINA (Berti, deGalperin, Pascual); BELGIUM (Vyt); FRANCE (Pêcheux); ISRAEL (Maital); ITALY (Venuti and Lis); JAPAN (Azuma, Ogino, and Toda); KENYA (Bali)

LAB/BRANCH
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experiences in infancy are acknowledged to affect the course and outcome of social and mental development in childhood, and they are credited for some of the distinctiveness of culture. More specifically, cross-cultural developmental studies have historically shown that variations in childrearing styles typically have implications for children's later cognitive performance and interpersonal behavior. Home environments are thought to reflect larger cultural values, beliefs, and customs, and many social theorists have contended that the family generally, and the mother-infant relationship specifically, may be vital to development of the individual and basic to the organization of the culture. As a result, investigators have frequently studied infancy and mother-infant interaction in attempts to address questions about the origins and development of cultural identity. Of course, each society has evolved patterns of childrearing adjusted to its own special demands. For example, it is widely held that Japanese and Americans differ in prominent aspects of their psychological make-ups and that certain social and intellectual distinctions between members of these two cultures arise early in life. Previous study on the nature of infant development in Israeli kibbutzim determined that many decisive aspects of infant care -- particularly the close ties between infant and mother -- vary markedly from the typical American experience. Contemporary France, Italy, and America are relatively similar in terms of industrial level, educational attainment, and living standards, yet these societies differ considerably in terms of history, sociology, and culture. Within-culture comparisons are equally important: Studies of development in Belgium offer answers to the question of how being reared in monolingual versus bilingual environments affects cognition, communication, and socioemotional development. Argentina contrasts middle-class with extremely poor rearing conditions, all in a South American setting virtually unresearched. What differences exist in parenting and in infant activity within and across these cultures? A central purpose of this project is to identify similarities and differences in the childrearing ecologies of Argentine, Belgian, French, Israeli, Italian, Japanese, Kenyan, and U.S. American children.

Project Description:

Objectives:

Certain caregiver activities have both immediate and protracted consequences for children's development in the first years of life. Most cross-cultural work investigates early infancy using samples of convenience. In this project, research focuses on the extent to which caregiver characteristics associated with particular cultures influence observed relations between maternal interaction patterns on the one hand and the development of children's culture-specific and culture-general social and cognitive competencies on the other. The study promises to be of great theoretical interest because potentially contrasting styles of childrearing can be directly compared while minimizing socioeconomic and educational differences, because of known international differences in children's preparation for school, and because the childcare arrangements in different traditions offer critical perspectives on early human development.

Considerable controversy has surrounded the ramifications of different caregiving arrangements for children's growth, yet few systematic, prospective longitudinal cross-cultural studies of adult parenting and infant behavior exist. Specific experiences at specific periods in infancy are thought to exert control over specific aspects of cognitive and social growth. Studies that have explicitly or implicitly adopted this view can be seen to have provided the most useful data on questions of early effects. These findings lead to various lines of research -- one to document similarities and differences in adult-child dyads in different cultural ecologies, and a second to determine the implications of these different ecologies for children's later behavior and performance. This research project is designed to pursue answers to these questions.

The reasons to conduct cross-cultural developmental research are many. One is description: Insofar as cross-cultural developmental descriptions of structure and process attempt to encompass the widest spectrum of human variation, they are also the most comprehensive in social science. They are vital to delimiting the full range of human experience. In this sense, too, they are critical to establishing valid developmental norms. Our awareness of alternative modes of development also sharpens our perceptions and enhances our understanding of the nature of our own culture. A second reason is explanation: Crossing cultures can provide unique insights in the quest for understanding forces that determine course and outcome in human development. Only the cross-cultural view can expose variables which regulate development, but which may be "invisible" from a monocultural perspective. In particular, this approach can help toward understanding parts which culture-dependent and culture-independent forces might play in the emergence and development of psychological structure and process. Further, cross-cultural study can occasion an unconfounding of variables thought to influence development, but which are normally compromised by monocultural investigation. The third reason is hypothesis testing: Cross-cultural developmental inquiry provides natural tests of special circumstances that might surround development, and it is critical to exploring cultural uniformity versus diversity of psychological and other social scientific constructs. A major purpose of this project is, therefore, to cross-validate and to extend previous work investigating the influences of caregiver behavior on infant development. Clearly, many of the reasons that motivate cross-cultural developmental research are descriptive, but salient ones also concern mechanism and process. All these motives at base help to explain adaptation in child development. This project is, therefore, designed to specify the nature of child-mother-caregiver relations among families

living in different normal urban environments and in different atypical environments.

Methods Employed:

This cross-cultural project is fully integrated with the domestic U.S. study as described in Z01 HD 01119-08 LCE, where the protocol is explained in greater detail. Notably, both studies capitalize on the same prospective longitudinal design and measures to evaluate child development and parent behavior across the first 4 years of life. Although the core of the research protocol is constant, some variations have been included in response to culture-specific research interests, as well as requirements of specific societies. Across all collaborations, moreover, cohorts of mothers and babies are being seen at 5, 13, 20, and 48 months.

Most participating cultural groups have been selected to be similar in terms of modernity, urbanity, education, and living standards. In each, the family is typically nuclear in organization; the mother is normally the primary caregiver in the family setting; and parents hold many of the same larger goals for their children, notably social adjustment, educational achievement, and economic security. Therefore, it has been possible to obtain samples from different cultures that are roughly equivalent in sociodemographic terms. At the same time, substantial differences exist among these cultural samples in terms of history, beliefs, and values associated with childrearing. As they are described, it is important to note that, in conjunction with these cross-cultural analyses, selected and meaningful within-culture comparisons are also being undertaken.

As with the choice of U.S. comparison groups in the domestic project, diverse rationales underpin the selection of particular cultural samples in the cross-cultural project. The reasons to compare and contrast childrearing in Japan and America are compelling. Briefly, contemporary Japan and the U.S. are alike in many sociological respects and, importantly, share many of the same child-centered goals. At the same time, Japanese and Americans are thought to differ markedly in childrearing styles. Japanese and American adults differ in salient aspects of their psychological make-up, and it is believed that certain of these social and intellectual differences arise early in life. The comparison of Japanese and American mothers in interaction with their preschool-age children is also valuable because of findings concerning possible familial antecedents of differential cognitive development in the two societies. Cross-national studies have shown that Japanese youngsters, before first grade, consistently outperform their American counterparts in academic achievement. This is so even though IQ per se does not apparently differ between children in these societies. The fact that differences in achievement obtain among preschoolers suggests that forces in the family, perhaps centering around mother-infant interactions, may influence their course. In this comparison, activity and interaction patterns which are distinctive to, as well as those which are similar between, these two disparate cultures are identified, and their predictive validity assessed.

An Israeli sample includes urban families as well as families from kibbutzim. On traditional communal kibbutzim in Israel, infants do not live with their parents, but grow up in "infant houses" where they play and eat with age-mates and are cared for by professional caregivers (metaplot). Normally women on these kibbutzim work full time, and the socialization of children is delegated to the community and to multiple caregivers rather than solely to parents.

Infants spend time with their parents at random periods during the day and each evening. Because of these circumstances, studies of kibbutz childrearing practices have long been considered instructive for the insights they may provide about the effects of early experience on human development generally, and for the light they may shed on childcare experiences in the U.S. specifically. The kibbutz as a research location permits exploration of provocative questions of differential caregiving effectiveness, because mothers and metaplot typically adopt different balances in basic ideologies of childrearing by emphasizing, respectively, nurturance and affection versus education and discipline. One factor that makes the Israeli situation unique is the relative equality of social status between parents and childcare workers, a situation quite different from the one in the U.S. where childcare workers are typically of lower education and economic level. Clearly, the kibbutz provides an extreme if not arresting variation of substitute caregiving experiences for children and on this basis constitutes an appealing research setting. Urban-dwelling Israeli nuclear families provide ready normal controls and a basis for cross-cultural comparison.

Argentina is a still-developing country plagued by economic instability. During the period of this study, Argentina experienced hyperinflation -- in July 1989, for example, inflation rose at a rate of four percentage points a day -- with severe consequences for standards of living. Some indicators that reflect this situation included a drop in real wages, rise in the cost of living, decline in citizen confidence, and increase in unemployment -- in all, a disorganization of the social fabric. Such radical cultural variation presented an opportunity to revisit many assumed factors and relations in the ecology of normal child development, such as the generally positive association thought to obtain between socioeconomic status and parenting. One objective of this natural experiment was, therefore, to analyze relations between SES and measures of maternal behaviors and perceptions about childrearing on the one hand and child outcomes on the other. (It seems important to point out as well that one of the major criticisms of contemporary psychology is its Eurocentrism. As a counterweight, Argentina represents a Latin American, Hispanic culture with sufficient similarity in terms of modernity, education, and so forth to populations studied domestically to permit comparisons, but at the same time a population in which child development is underresearched, if not wholly neglected.)

The nature of communicative, cognitive, and social competence can be specially studied, and unique insights into child language development may be gained, through examination of children's linguistic development in the cognitively challenging situation of the bilingual environment. Although constitutionally one nation, Belgium is in fact two countries in one. Northern Belgium is Flemish in language (ca. 60%), whereas southern Belgium is Walloon French-speaking (ca. 40%). A border exists between the two communities -- although called the "Language Frontier" it is, of course, far more than this. The situation along and above the Belgian Language Frontier makes the study of developmental consequences of exposure to bilingualism a natural one. Most such research focuses on sequential bilingualism in preschool and school-age children (second language acquisition); the focus of this research effort falls on simultaneous bilingual experience from infancy. Infant exposure to bilingualism can be defined as a child regularly hearing two or more languages from birth. Earlier views had it that children with multilingual heritages were disadvantaged in language development. Today, it is believed that the advantages of bilingualism outweigh the disadvantages in, for example, control of processing and analysis of knowledge. Belgium affords a special experimental setting in which to conduct studies of early bilingualism. Bilingualism in Belgium is inherent to the

cultures of the country. It is positively promoted; it is also less sequential, less bound to SES, and early developing. Like others in this project, the Belgian samples are also middle-class, and so simultaneously permit cross-cultural comparison (and the use of U.S. data as a point of reference for the monolingual Flemish group). Finally, these Flemish monolingual and Flemish-French bilingual data sets can be joined with a monolingual French data set (existing in this laboratory) to effect unique three-setting longitudinal studies of language development.

Although parts of one nation since 1861, the North and the South of Italy nevertheless have many historical, cultural, and social differences. From an historical point of view, the South grew under Spanish influence and has maintained an economy and social organization typical of a rural society. Administration is centralized in two big towns (Naples and Palermo) in striking contrast with a majority of small villages. The North of Italy developed under the influence of Central Europe, particularly Austria and France, has prospered on trade and advanced manufacturing techniques, and has developed mostly into industrial cities with autonomous administrations. Our research is being conducted in the Veneto region in the North and in the Basilicata region in the South, in the areas of Padua-Venice and surrounding the village of Ruoti, respectively. This choice represents a classic Italian North-South comparison. These two regions differ importantly in terms of typical family structure, which is strictly patriarchal in the South versus more egalitarian in the North. In contrast with the North, women in the South normally do not work outside the home. There, the education of children is a wholly feminine task, children are rarely separated from their mothers, and fathers and babies are seldom in contact. Moreover, social services and support structures for family and children are nonexistent in the South, and school children are especially disadvantaged. These strong regional differences in the same country suggest a study of parenting in the context of controlled but contrasting family life styles and structures to obtain experimental evidence on the roles of cultural and social factors on parenting and development.

Current status. In Argentina, a total of 89 infants, 58 of middle SES parents and 39 of low SES, comprise the sample observed in collaboration with Dr. C. Galperín of the Universidad de Belgrano. In addition, 39 infants of low SES have been observed in a rural community outside the interior city of Córdoba. In Belgium, 30 monolingual and 24 bilingual mother-infant dyads have been observed in collaboration with Dr. André Vyt of the University of Ghent. In France, 46 French mother-infant dyads have been observed in collaboration with Dr. M.-G. Pêcheux of the Sorbonne. In Israel, A total of 60 infants, 30 living in greater Haifa and 30 living on traditional kibbutzim, have been observed with their mothers and metaplot in collaboration with Dr. S. Maital of the University of Haifa. (Infants living on kibbutzim were observed on two occasions: In the first, infants were seen individually with their primary metapelet; in the second observation, which took place within one week of the first, infants were seen individually with their mothers in a similar situation at home. Urban infants were observed once, with their mothers.) In Italy, a total of 86 infants (so far 56 from north Italy and 30 from south Italy) have been observed in collaboration with Dr. Paola Venuti in collaboration with Dr. Andriana Lis of the University of Padua. In Japan, two cohorts have been seen (a total of 48 infants living in Tokyo) in collaboration with Dr. M. Ogino of Sophia University, Dr. H. Azuma of Shirayuri College, and Dr. S. Toda of Hokkaido University. In addition, a sample of 30 5-month-old infants and their mothers has been recruited in Kenya by Dr. Shashi Bali of Kenyatta University.

Strategy for analyses. Data collection is underway in several of these cross-cultural samples. Planned cross-cultural analyses parallel those being carried out in the U.S. longitudinal sample. Some comparisons address questions of stability, experiential effects, and parenting within cultures. Others examine the origins and development of contrasting cultural styles, notably issues of cross-cultural variation and the nature of coherence in maternal ideas and activities, the composition of maternal responsiveness, emphases of maternal speech to infants, correspondences in mother-infant interaction, and styles of mother and toddler language and play.

Major Findings:

Cross-cultural comparisons. Psychosocial predictors of Argentine and U.S. mothers' decisions to work and amount of hours worked (in working mothers) were examined. Years of marriage and months that mothers worked during pregnancy predict whether or not mothers are employed after childbirth in both settings. By contrast, when the amount of hours that mothers worked was analyzed, different results were found in the two countries: Better educated women with higher status occupations work longer hours after childbirth in the United States, whereas better educated women with higher status occupations work fewer hours in Argentina. Different cultural and economic conditions in the two countries appear to mediate psychosocial determinants of women's decision to work.

The vocalizations of infants from three linguistic groups -- French monolingual, Flemish monolingual, and bilingual French/Flemish -- have been compared in a preliminary way for syllabic (production of speech-like and other syllables types) and prosodic features (analysis of prelinguistic phrasing). A first analysis of cross-linguistic comparisons at 5 months showed that the overall vocal production of the infants across groups is consistent with previous studies (about 3.5 syllables per minute, 2 utterances per minute, and slightly fewer than 1 phrase per minute across all of the samples). A finding emerged when group comparisons were conducted on the syllables per minute and utterances per minute variables. In this analysis, the bilingual group produced about 35% more vocalizations than did the monolingual groups for syllables per minute and for utterances per minute. The monolingual groups were equivalent to each other in vocal output. Infants in all of the groups produced substantially fewer canonical syllables than other syllables (the groups produced about 3% canonical syllables, 0.5% marginal syllables, 34% fully resonant nuclei, and 56% quasiresonant syllables), and these data are consistent with what we already know about vocal production at 5 months, but they will be valuable because they will provide additional information on cross-linguistic patterns. Again, the bilingual group produced a substantially greater percentage of canonical syllables in their vocalizations than did the monolingual groups. The infants in the bilingual environment talked more and produced greater percentages of developmentally advanced syllable types than did the infants in the monolingual environments.

Activities and interactions of Israeli and U.S. American mothers and their 5-month-old infants were observed in the natural setting of their homes, including infant visual and tactual exploration and vocalization as well as maternal stimulation and speech. Similarities and differences in activities between Israeli and U.S. infants and mothers were assessed. Coherence in infant activities and in maternal activities within each society was evaluated, and resultant

patterns of coherence between the two societies compared. Last, correspondences between infant and maternal activities in each society were analyzed, and resultant patterns of mother-infant interactions between the two societies compared. Identification and description of activities, interactions, and developmental processes which are similar and different in comparable segments of Israeli and U.S. society were explored, and cross-cultural tests of developmental issues related to coherence and to correspondence of activity in mother-infant dyads evaluated. Israeli and U.S. mothers were found to follow culture-specific paths in striving to meet infants' needs and in achieving socialization goals.

A second study explored cross-cultural differences in maternal teaching styles during joint picture-book reading by U.S. and Israeli mothers and their 4-year-olds. Maternal behaviors included references (links) to the child's past unique and routine experiences, anticipated future events, dyadic shared knowledge, maternal inference demands, and reality checks. Several multiple hierarchical regression analyses, with maternal bookreading style measures as the dependent variables, and controlling for maternal education and child Verbal IQ, suggested that maternal styles of bookreading vary with culture, with U.S. mothers adopting a more didactic style than Israeli mothers.

During the course of this study, Israeli mothers' reports of their own and their toddlers' stress reactions to the Gulf War were collected and compared with those of American mothers and toddlers. It is unusual to be able compare reactions to war cross-culturally. However, in an age of global politics and telecommunications, war touches distant populations. Participation of American armed forces in the Gulf War created a sense of crisis among many Americans as well as among Israelis. Because young children's reactions may reflect their mothers' responses as well as their awareness of the situation, we investigated the responses of both mothers and their children and the children's awareness of the war. Israeli and American mothers of 2- to 3-year-old children evaluated their own and the toddlers' responses to stress. Results showed few differences between groups in stress reactions, despite differences in children's awareness of the war. In both groups, maternal stress related similarly to children's coping.

Central to a concept of culture is the expectation that different peoples possess different ideas and behave in different ways with respect to childrearing. In one study, ideas that Argentine, French, and U.S. American mothers hold about their own and their husbands' actual childrearing were investigated, as well as what mothers consider to be ideal childrearing, in three domains: social, didactic, and disciplinary parenting. For each domain, we analyzed mothers' reports of their actual behaviors and of their husbands' actual behaviors; mothers' ideal expectations of their own and their husbands' behaviors; and mothers' dissonance with respect to parenting, i.e., the extent to which mothers' actual and their husbands' actual behaviors each agrees with mothers' ideal expectations for themselves and for their husbands. The results showed consistent parent, country, as well as parent-by-country effects interpretable in terms of overarching cultural beliefs. Understanding parents' ideas contributes to understanding why and how parents behave toward children, and provides insights into the broader social context of child development.

Certain psychological characteristics arise early in life, and the nature of parent-child interaction is thought to constitute at least one important source of individual development as well as cultural variation. Two theoretical reviews explored formative experiences parents provide young children that relate to the origins and development of contrasting cultural styles through an

examination of families from different Western and Eastern cultures. One discussed cross-cultural variation and the nature of coherence in maternal activities, responsiveness, and speech as well as correspondences in mother-child interactions. In the second, understanding of parenting and development and their meaning was viewed through the prism of context. A given activity often has the same meaning in different contexts, but one activity can also have different meanings across contexts. Conversely, different activities can have different or similar meanings depending on context. A prime context for discussing relations between activity and meaning is culture. Flexible relations between activity and meaning were examined in a unified conceptual scheme, and illustrated with phenomena from culture and human development. Both the heuristic value of this perspective and its implications for studies of culture and human development are also considered.

Within-culture comparisons. An intra-cultural study was conducted within Argentina. The ideas that Argentine mothers hold about childrearing were investigated, comparing mothers of different socioeconomic levels and urban and rural residence. Specifically, three domains of childrearing were studied: social, didactic, and discipline. For each domain, mothers' perceptions of their actual and ideal behaviors and of their husbands' actual and ideal behaviors, and mothers' satisfaction with their own and their husbands' behaviors were analyzed. The results showed significant differences between mothers' ideas about their own and their husbands' behavior, specially in the social and didactic domains. Socioeconomic level and place of residence had limited effects on mothers' ideas about parenting.

Two samples, on from the North and one from the South in Italy are being studied. In an effort to understand better the "culture of the South", a small conference was convened: "Lo Stile Dell'Interazione Madre-Bambino in Differenti Contesti Culturali Italiani". In a preliminary analysis, maternal speech to 13-month-olds has been compared between the two in two different ways, under the hypothesis that speech addressed to older infants might differ selectively in content and form depending on language socialization preferences. Mothers in the North and South differ in age, educational level, and SES. Two general classes of maternal utterances were first defined -- features concerned with affect versus features concerned with communicative competence; within each class, a number of subcategories were delineated. Mothers in both locales used these speech categories to their infants; however, they were culturally differentiated with respect to what they emphasized in their speech. For example, mothers in the South displayed higher frequencies of affect-salient speech than mothers in the North. A second analysis used another approach to transcript coding. The amount of speech in the North was more than the amount of speech in the South (as might be expected from the differences in both maternal education and SES favoring the North), even though the amount of time recorded was equal for each. As a consequence, all subsequent analyses were conducted on the basis of proportions. Correlations between, on the one side, age,
SES, and maternal educational level on the part of mothers and, on the other, language categories
showed that (for both frequency and proportion) there are expected positive correlations between, for example, SES and educational level and maternal tutorial and conversation and negative correlations with control and asynchrony. So results on demographics quite well match expectations. Third, ANCOVAs looking at site (North and South) and sex (boys and girls) covarying (successively) maternal age, SES, and education on the proportion data for each category showed that, even with the significant differences in the sociodemographic variables

covaried, mothers in the North use proportionately more didactic speech and marginally more conversation, and mothers in the South do significantly more control. Having controlled for SES, education, and age of the mothers, it is likely that these differences represent residual "intracultural"/"stylistic" differences in the North and South of Italy. Interestingly, didactic speech is a longitudinal predictor of child language, and the fact that there is more didactic in the North than in the South opens up an additional point of exploration. In this way, maternal speech to babies appears to emphasize different cultural agenda.

Significance of Biomedical Research and the Program of the Institute:

The Section on Child and Family Research (SCFR) was established with the aim of pursuing research on the ways in which children's development is affected by variations in the conditions under which they are reared. Some of these variations are studied in the U.S., where the Section examines aspects of the quality of care, family organization, child temperament, and the like (Z01 HD 01119-08 LCE). In many cases, however, the SCFR's understanding of child development is enhanced by conducting research in cultures outside the U.S., because the variations in rearing conditions are greater and the specific variations are of theoretical importance. Moreover, cross-cultural perspectives help to define norms of human development and help to specify contexts of development and their influences on parents and children more accurately and comprehensively.

Proposed Course of Project:

Data collection and analysis of Argentine, Belgian, French, Israeli, Italian, Japanese, Kenya, and United States samples continue. Recruitment of additional cultural samples is underway.

Publications:

Bloch, H, Vyt, A, & Bornstein, MH. An introduction to Francophone research and thinking in developmental psychology. In: Vyt A, Bloch H, Bornstein MH eds. Early child development in the French tradition: Perspectives from contemporary research. Hillsdale, NJ: Lawrence Erlbaum Associates, 1994;1-12.

Bornstein, MH. Francophone perspectives on cognition and social cognition in preschool-age children. In: Vyt A, Bloch H, Bornstein MH eds. Early child development in the French tradition: Perspectives from contemporary research. Hillsdale, NJ: Lawrence Erlbaum Associates, 1994;171-3.

Bornstein, MH. Form and function: Implications for studies of culture and human development. Cult & Psychol 1995;1:123-37.

Bornstein, MH. La relazione madre bambino in una prospettiva cross-cultural; Somiglianze e specificità culturali in differenti contesti: Americhe, Giappone, Europa. [Mother-infant relationships in cross-cultural perspective: Similarities and specifics in different cultural settings in the Americas, Japan, and Europe]. In: Giusti F, Venuti P eds. Le relazioni familiari evoluzione, biologia, cultura (pp. xx-xx). Rome, Italy: Giunti, 1995.

Bornstein, MH, Maital, S, Tal, J, & Baras, R. Mother and infant activity and interaction in Israel and in the United States: A comparative study. Inter J Behav Dev 1995;18:63-82.

Bornstein, MH, Tamis-LeMonda, CS, Pascual, L, Haynes, OM, Painter, K, Galperín, C., & Pêcheux, M-G. Ideas about parenting in Argentina, France, and the United States. Intern J Behav Dev 1995;

Maital, S, Bornstein, MH, Andreassen, C, & Tal, J. T'guvot shel imahot V'yaldehen hatzi-erim l'Milchemet Ha 'Mifratz': Hashva-ah bein Yisrael V'Artzot Habrit. [Reactions of mothers and their young children to the Gulf War: A comparison of Israel and the United States.] Psychologia 1994;4:207-213,221.

Pascual, L, Haynes, OM, Galperín, CZ, & Bornstein, MH. Psychosocial determinants of whether and how much new mothers work: A study in the United States and Argentina. J Cross-Cult Psychol 1995;26:314-30.

Pascual, L, Schulthess, L, Galperín, CZ de, & Bornstein, MH. Las ideas de las madres sobre la crianza de los hijos en Argentina [Ideas of mothers about child development in Argentina]. Revista Interamer de Psicol/Interamer J Psychol 1995;29:23-38.

Vyt, A, Bloch, H, & Bornstein, MH eds. Early child development in the French tradition: Perspectives from contemporary research. Hillsdale, NJ: Lawrence Erlbaum Associates, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 01122-08 LCE

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Longitudinal Assessment of Children's Mental and Social Abilities

PRINCIPAL INVESTIGATOR *(List other professional personnel below tha Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD

COOPERATING UNITS *(if any)*

Infancy Studies Program, New York University (Tamis-LeMonda)

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Section on Child and Family Research

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research continues a longitudinal study of mental and social development in two cohorts of children observed repeatedly between 2 months and 4 years of age. The premise of this research is that learning in childhood occurs primarily within an interpersonal context; thus, a central goal is to describe how diverse infant and maternal behaviors relate to the ontogeny of central dimensions of children's mental and social competencies. Children participating in the assessment constitute longitudinal cohorts seen at 2, 5, 13, 20, and 48 months of age. Major objectives of this research are to study stability, continuity, and interaction in mental and social development over the first years of life.

Project Description:

Objectives:

This project proceeds from the theoretical idea that learning in young children occurs in social contexts. The studies encompassed by the project are designed to explore maternal contributions to cognitive and social development in infancy and early childhood by examining a wide range of parenting activities, including the teaching strategies mothers adopt when structuring their children's learning. A major objective of this longitudinal research is the study of stability in development and the contributions of early experience and infant behavioral style to later capacities. Mothers and children have participated in this longitudinal study at several points over the first four years of their children's lives. Assessments of behavioral, cognitive, social, and temperament variables have been conducted on children and mothers alike. In addition, mothers of are tested for their sensitivity to children's cognitive level and for their selection of teaching strategies.

Methods Employed:

This research examines interrelations among dimensions of infant cognitive abilities, temperament, behavior, social interaction styles, and mother-infant interaction in a sample of approximately 50 mother-child dyads seen when children were 2, 5, 13, 20, and 48 months of age.

Major Findings:

This phase of the research examined interrelations among dimensions of infant ability and style and mother-child interaction over 2-, 5-, 13-, and 20-months. Several noteworthy results have been obtained from these analyses.

Two integratrive reviews of information processing were written by invitation. In one, matters involved in the study of habituation's role in mental development from early infancy were assessed. First, the method of infant assessment was described: Habituation is the decline in infant attention to stimulation that is available continuously or is presented repeatedly. Second, measurement studies of procedural and psychometric characteristics of habituation show individual differences and adequate short-term reliability. Third, evaluations of prominent models of stability from habituation in infancy to measures of cognitive performance in childhood are evaluated. Fourth, arguments for the meaning of habituation as an indicator of infant cognitive functioning are assessed on sundry bases, including notably its predictive validity. On several arguments, this capacity is interpreted as indexing infant "information processing," and habituation likely plays a role in the expression of childhood cognitive performance. Finally, habituation in at-risk cocaine-exposed infants is explored, and its meaning evaluated. A companion chapter reviews some procedural and psychometric issues involved in the measurement of habituation, examines arguments for the information-processing interpretation, surveys data for the predictive validity of habituation in normal infants, and discusses the origins of habituation as well as the role of experience in its expression. Finally, habituation in at-risk cocaine-exposed newborns and young infants is described.

The start of the second year is a period of transition and substantial variability among toddlers in language, play, and attention abilities. A series of studies examined toddler abilities and maternal parenting activities in relation to those abilities. First, relations among specific aspects of language (comprehension and production, semantics, and utterance length) and relations between language and symbolic play were evaluated at 13 and 20 months and longitudinally between 13 and 20 months. The contributions of maternal stimulation to toddler performance, and whether associations among toddler abilities might be explained by maternal behaviors, were also examined. Diverse measures of toddler language covaried; language-play associations in toddlers were specific to semantic aspects of language. Associations between mother and toddler behaviors emerged, and they also tended to be specific: Maternal language related to

toddler language, and maternal play related to toddler play. Moreover, relations among toddler abilities maintained after maternal influences were partialled. These findings were examined with respect to the multidimensional structure of language and specificities in language-play associations in children's representational development.

Three papers examined diverse aspects of early language development. In one chapter, an empirical update about some early ontogenetic aspects of language acquisition in provided. Select recent research is described on how infants develop from nonverbal individualists into interactive conversationalists, willing and able to articulate their cares, needs, desires, and dreams to others. The chapter starts with a brief description of norms and individual variation in early language acquisition as background, and then turns to discuss how infants' experiences complement their capacities by channeling the development of communication toward increasingly effective interactions and integration into the social world. Next, production and comprehension in two important and basic systems of language, perception and production of speech sounds and how children begin to acquire referential forms, are reviewed.

For both mothers and researchers, infant vocal distress must fit into a conceptual structure of infant behavior in general. The first section of the second paper reviews the methods and results of a study of mothers' and observers' conceptions of infant vocal distress vis-à-vis other salient infant behaviors. The second and third sections explore how mothers' and observers' conceptions of infant distress vocalizations affect aspects of their behavior directly related to the cry.

The third chapter concerns children's early vocalizations and language development. First, longitudinal and cross-cultural measurements of infant vocalizations in the first year are reported; factors that influence infant vocalization are explored; and effects of infant vocalization are described. The second part reviews data on stability and change in, and mother-child interactional influences on, children's language performance in the second year. In the third part, nonlinguistic factors that affect language development from infancy, including infant information-processing abilities, maternal interaction styles, and infant effects are discussed. A consistent theme in this chapter is the significance of individual variation in child and parent for child language study.

Researchers and theoreticians speculate about the nature and correlates of children's early play. What is play like? How does play change over the course of childhood? What inner abilities might play reflect?

One investigation of mother and toddler play had two goals: The first was to describe maternal scaffolding behaviors in play interactions by examining the types of play mothers introduce in response to their toddlers' play, and the second was to examine the role of maternal knowledge about child play in maternal scaffolding. Fifty mothers and their 21-month-old toddlers were observed at home in free play. Three levels of maternal play (i.e., exploratory, nonsymbolic, symbolic) were coded with respect to toddler play. Sequential analyses addressed the first goal and revealed that mothers adjusted the level of their play to match or advance their children's play level. A second set of analyses revealed that mothers who were more knowledgeable about the development of child play responded to their children's play with more play at higher levels than their children's play. Thus, mothers as a group play with their toddlers in psychologically meaningful ways, and mothers with more knowledge about child play development provide their children with appropriately challenging play interactions.

We have also examined maternal knowledge and behavior with respect to children's play. Mothers of 21-month-olds were given a list of 24 child play acts and asked to rank order acts in terms of relative difficulty. Included were exploratory behaviors (e.g., mouthing), nonsymbolic acts (e.g., nesting cups), and symbolic acts (e.g., drinking from an empty cup). In general, mothers' rankings matched scalings of play established in the developmental literature. These findings represent a first step in elucidating mothers' knowledge about development in the domain of child play.

In addition, two reviews of play and its development were undertaken. In the first, methodological approaches and assessment procedures used in the study of child play were reviewed, and a general model of play was documented.

In symbolic play, children construct increasingly sophisticated representations of the world as well as relations between symbols and their external referents as they internalize, consolidate, and advance upon their developing cognitions about people, actions, and objects. Presumably, more sophisticated partners, like parents, promote children's development in this domain of action. Yet, the empirical literature to date shows little support for the notion that child symbolic play grows through adult-child interaction. The second theoretical paper reviewed empirical studies that address the role and effects of a more sophisticated partner on children's early symbolic play, then presented three theoretical perspectives that support a view that symbolic interactions with adults ought to promote children's symbolic play and advance children's representational competencies more broadly; they include attachment, scaffolding, and ethological theory. Finally, the paper revisited the literature on interactive influences on children's play reconsidering the nature and role of specific independent and dependent variables in studies of the growth of children's symbolic play.

Several theoretical and review papers on mother-child interaction and responsiveness, as well as stability and continuity in development appeared this year in connection with this research. Multiple considerations of infancy provided a common framework for many of these papers. Infancy is remarkable because of the dramatic developmental changes that take place in this short period and because of the influence experiences in infancy can exert on later development. The first years witness significant advances in the shape and capacity of the body and its muscles, substantial growth in complexity of the nervous system, rapid development of sensory and perceptual abilities, major milestones in understanding and mastering the object world, acquisition of basic language skills, formation of significant social bonds, and emergence of characteristic personal and social styles. At no other point in the life span do so many aspects of the individual change so rapidly and so fundamentally.

How caregivers respond to infant signals and development and what consequences, if any, caregiver responsiveness has for the child are issues that are examined in a separate theoretical treatment. First, what responsiveness is and what its typical characteristics are were defined. Next, what effects responsiveness has on infant crying and what short- and long-term effects it has on infant cognitive development were examined. Then, how responsiveness operates in growth and where responsiveness in the caregiver comes from were discussed. Many research reports converge in showing the powerful role that responsiveness plays in the development of a broad range of child competencies in development. To provide this kind of responsiveness, caregivers need to know each child in their care, and they can achieve this through developing a close relationship and being attentive and receptive to each child's signals. Responsiveness is a deceptively uncomplicated scenario. Hidden, however, are the telling and powerful consequences responsiveness portends, not only for the infant's survival, but for the positive development of intellectual capability and social self-confidence in the child.

Finally, two invited pieces revisited issues of stability and continuity in mental development. In one chapter, we first reviewed samples of stability research, underlying visual information processing and understanding causal relations. Next, we discussed briefly some possible antecedents of these abilities in infancy and then some possible mechanisms underlying stability. Finally, we considered some compelling questions left still unanswered in this developmental work and implications of these findings for theories and models of intellectual development. Alfred Binet co-founded L'Année Psychologique, the first French psychological journal, which he edited for its first 17 years. For Le Centenaire de l'Annee Psychologique, a paper was prepared reviwewing Alfred Binet's life, his work on the origins of mental testing, and its consequences for understanding the development of human intelligence.

Significance for Biomedical Research and the Program of the Institute:

The scientific yield of longitudinal assessments of development holds great potential in itself as well as being tailored to the special needs of future NICHD research projects. In addition, the longitudinal nature of this project is spelling out critical aspects of parenting involved in the early development of mental and social functioning in children's first years of life.

Proposed Course of Project: Analyses of these data are ongoing. First, the degree of independence/interrelatedness among child cognitive and social measures is being determined in conjunction with the evaluation of the independence/interrelatedness of these measures with other data sets. This is an iterative process -- results are reviewed and new approaches proposed which, in turn, are submitted to additional evaluations -- and substantial work investment and judgment are required to arrive at a proper understanding of the findings. Predictive relations between observations of these dyads during the first years of life and performance at 4 years are planned. Analyses of the implications of first- and second-year experiences for later preschool development continue.

Publications:

Bornstein, MH. Alfred Binet, the origins of mental testing, and the consequences for understanding the development of human intelligence. In: Fraisse P, Segui J eds. Les origines de la psychologie scientifique: Centenaire de l'Année Psychologique. Paris: Presses Universitaires de France, 1994;145-61.

Bornstein, MH. Origins of communication in infancy. In: Velichovsky BM, Rumbaugh DM eds. Human by nature: Origins and destiny of language. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers, 1995;191-237.

Bornstein, MH, & Bornstein, HG. Caregivers' responsiveness and cognitive development in infants and toddlers: Theory and research. In: Mangione PL ed. Infant/toddler caregiving: A guide to cognitive development and learning. Sacramento, CA: California Department of Education's Bureau of Publications, 1995;12-21.

Bornstein, MH, & Bornstein, HG. Infancy. In: Bornstein MH, Bornstein HG, Landers C eds. Infancy and early child development. New York City: UNICEF, 1995;

Bornstein, MH, Bornstein, HG, & Landers, C. Infancy and early child development. New York City: UNICEF, 1995.

Bornstein, MH, Mayes, LC, & Tamis-LeMonda, CS. Habituation, information processing, mental development, and the threat of cocaine exposure in infancy. In: Hepper PG, Kendal-Reed M eds. Perinatal sensory development: Psychology and psychobiology. Cambridge, England: Cambridge University Press, 1995;

Bornstein, MH, Slater, A, Brown, E, Roberts, E, & Barrett, J. Stability of mental development from infancy to later childhood: New wine and old bottles. In: Bremner G, Slater A, Butterworth G eds. Infant development: Recent advances. Hove, England: Lawrence Erlbaum Associates, 1996;

Bornstein, MH, & Tamis-LeMonda, C. Antecedents of information-processing skills in infants: Habituation, novelty responsiveness, and cross-modal transfer. Inf Behav Dev 1994;17:371-80.

Bornstein, MH, & Tamis-LeMonda, CS. Language and nonlanguage factors in development of prelinguistic vocalization to linguistic communication. In: Konopczynski G ed. Is early language performance predictive of later language development? Calais, France: Ortho-Editions, 1995;

Bornstein, MH, & Tamis-LeMonda, CS. Parent-child symbolic play: Three theories in search of an effect. Dev Rev 1996;

Damast, AM, Tamis-LeMonda, CS, & Bornstein, MH. Mother-child play: Sequential interactions and the relation between maternal beliefs and behaviors. Child Dev 1996;

Mayes, LC, & Bornstein, MH. Infant information-processing performance and maternal education. Early Dev Parenting 1995;4:91-6.

Tal, J, & Bornstein, MH. Infant vocal distress: Conceptual structure, responsiveness, and sampling in mother and independent observer. In: Lester BM, Newman JD, Pedersen F eds. Social and biological aspects of infant crying. New York: Plenum, 1995;

Tamis-LeMonda, CS, & Bornstein, MH. Specificity in mother-toddler language-play relations across the second year. Dev Psychol 1994;30:283-92.

Tamis-LeMonda, CS, & Bornstein, MH. Variation in children's exploratory, nonsymbolic, and symbolic play: An explanatory multidimensional framework. In: Rovee-Collier CR ed. Advances in infancy research, Vol. 10. Norwood, NJ: Ablex, 1996;

Tamis-LeMonda, CS, Damast, AM, & Bornstein, MH. What do mothers know about the developmental nature of play? Inf Behav Dev 1994;17:341-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01123-05 LCE

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiological Correlates and Neural Mechanisms of the Infant Cry and Related Vocalizations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: John D. Newman Section Head, LCE:NICHD

Others:	Moody, Kathleen	IRTA Fellow	LCE:NICHD
	Norcross, Janet	IRTA Fellow	LCE:NICHD
	Smith, Najwa	IRTA Predoctoral Fellow	LCE:NICHD
	Bernhards, Deborah	Bio. Lab. Tech	LCE:NICHD

COOPERATING UNITS *(if any)*

Department of Zoology, University of Maryland at College Park (C.S. Carter); Yerkes Regional Primate Research Center (T. Insel); Wisconsin Regional Primate Research Center (D. Abbott)

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Developmental Neuroethology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has begun to explore the association between endogenous hormone levels and vocal behavior in the squirrel monkey. In one study, neither cortisol nor testosterone levels correlated with production of alarm calls in adult males. However, a weak correlation was found to exist between cortisol levels and alarm calling rate in adult females. Castrated males were found to have a lower response threshold and steeper dose-response (using rate of alarm calling as the response measure) in a behavioral pharmacology model for defensive threat reactivity. Castrated males also differed from intact males in vocal behavior during same-sex social interactions. The castrates made more twitters and chucks, while intact males made more err chucks and peeps. The former call types are associated with friendly interactions, while the latter are associated with excitement and arousal. New methodology for imaging histological brain sections were initiated, and the brains of adult male and female squirrel monkeys (a species with robust sex differences in behavior) were examined for quantitative differences in the size of forebrain areas.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

The major goal of this project is to understand the neurophysiological and neurochemical mechanisms and related brain substrates underlying the expression of vocalizations in nonhuman primates and the relationship of these mechanisms to those regulating the vocal expression of affect in humans. The present investigations use vocalizations as behavioral probes for assessing the potential clinical value of novel pharmacological agents, for assessing the behavioral effects of experimental neurological procedures, and for examining the relationships between vocal and nonvocal measures of emotional state.

Methods Employed:

Standardized testing paradigms involve the natural vocal response to brief social separation in squirrel monkeys (Saimiri), rhesus macaques (Macaca mulatta), and common marmosets (Callithrix jacchus), as well as the vocal alarm exhibited to threatening external stimuli. Quantitative, computer-assisted analyses of the production rate and acoustic structure of vocalizations in these tests provide objective assessments of subject response during experimental and control trials. Computer-based methods are used to identify specific vocalizations according to established diagnostic criteria and to measure acoustic differences related to experimental procedures. Neuroendocrine and neuropharmacological techniques involve behavioral assessments following administration of systemic and intracranial doses of peptide and nonpeptide hormones and drugs with activity at specific neurochemical receptor sites. Brain sites of peptide and drug action are determined using autoradiographic and immunocytochemical techniques.

Major Findings:

A. Autoradiographic demonstration of brain receptors for oxytocin in the common marmoset and squirrel monkey.

Receptor autoradiography studies of common marmoset brains are continuing with 3 adult males and 3 adult females currently being analysed to determine quantitative differences in the location and the number of oxytocin receptors in male and female brains. We have also begun to investigate squirrel monkey brains and currently have one adult male and one adult female brain to analyze for oxytocin receptor distribution.

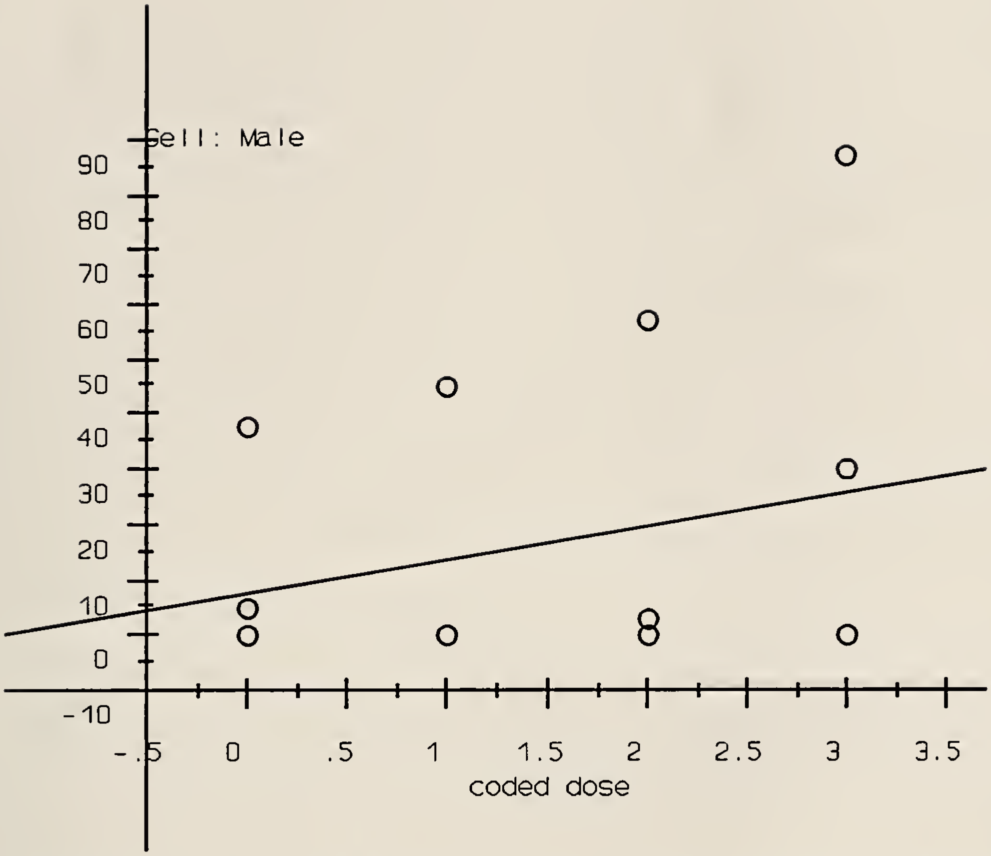
B. Immunocytochemical Demonstration of Neuropeptide Distribution in the Brain of Common Marmosets.

We have continued our investigation of oxytocin and vasopressin immunoreactive neurons in the brains of common marmosets. With respect to vasopressin immunoreactivity, we have thus far examined 4 adult males and 4 adult females and there does not appear to be a gender difference in the number of cells in the lateral septum, an area that shows gender difference in highly affiliative species of rodents. There is, however, a gender difference in the bed nucleus of the stria terminalis, the cells that project to the lateral septum. Studies are currently

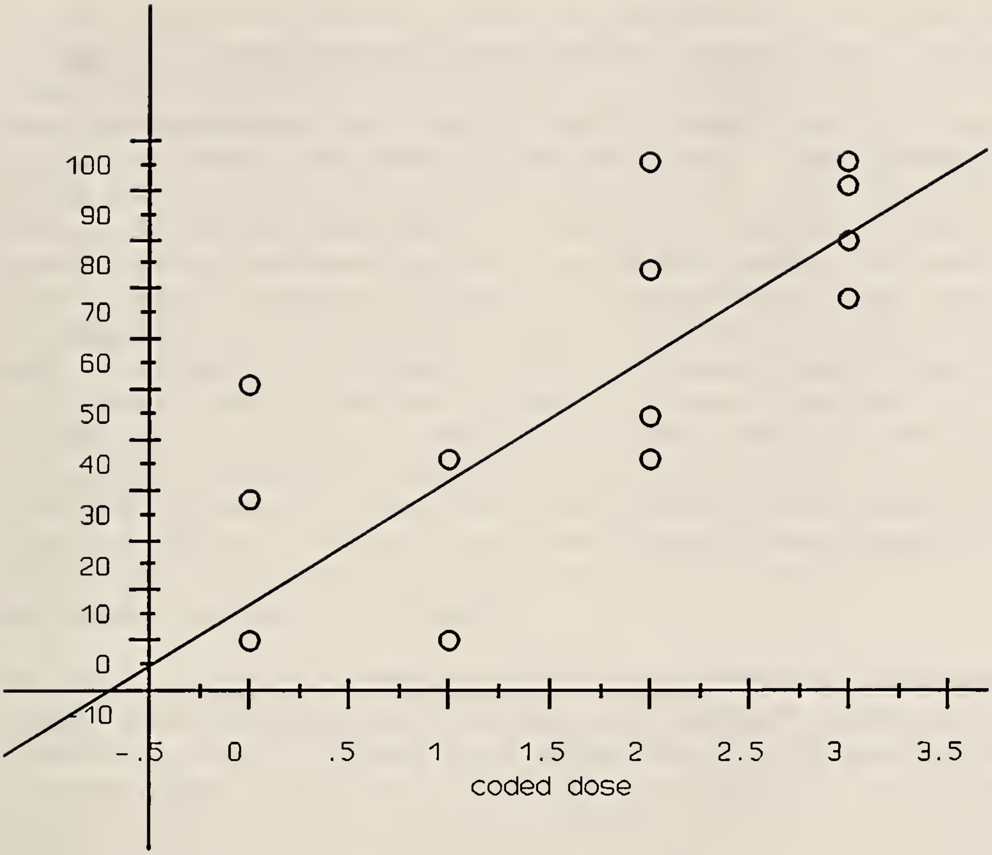
underway to more clearly define sex differences in these and other areas. With respect to oxytocin, no differences have been found between males and females in the paraventricular nucleus or the supraoptic nucleus of the hypothalamus, and studies are currently focusing on receptor colocalization studies to determine if oxytocin and/or vasopressin neurons contain estrogen or androgen receptors, which would indicate regulation of these peptides by steroid hormones.

C. Gender Differences in the Ethopharmacology of Anxiety-related Behavior in Squirrel Monkeys and Possible Hormonal Correlates.

Given the differences in strength of behavioral response to mild environmental challenge (presence of human wearing capture glove) in females, intact males, and castrated males reported last year, there is increasing interest in determining in greater detail the relationship between hormonal status and behavioral response in this paradigm. Eighteen naive adult squirrel monkeys (8 males and 10 females) were chosen for study, in addition to the 4 castrated males used in the previously reported experiment. Fertility of the females was assured, in that each had given birth within a year of the onset of this study. Plasma testosterone levels of the males were assessed at the start of the study, and again after the study was completed. The paradigm used in previous experiments was continued for this study; it involves capturing each subject, injecting drug (benactyzine hydrochloride) or vehicle (sterile water) subcutaneously, and transporting the subject to an observation cage situated in a quiet testing room. The subject was then left alone for 5 min (sufficient time to permit the drug to be carried to the brain). Testing consisted of the same 3 experimenters involved in capturing and injecting the subject entering the test room, and standing motionless, with a hand covered by a leather capture glove raised, next to the observation cage for 1 min (Test I). Behaviors measured were vocalizations (divided into 6 acoustic subtypes), locomotor activity, and visual scanning. Immediately following this 1- minute test, a second test was instituted, in which the small cage used to transport the subject between its home room and the test room was attached to the side of the observation cage, and the experimenters again stood motionless for 1 min, but without the capture gloves (Test II). Pilot studies revealed that this testing procedure often elicited a stronger "alarm" reaction than the first test. At the end of testing, the door to the transport cage was raised, permitting the subject to enter, after which it was returned to the colony. All subjects were tested between 10:00 am and noon once per week. Males and females were tested on different days. As found previously, a significant difference in response strength between females and intact males occurred, but only at a drug dose of 0.1 mg/kg. A significant difference in response strength also existed between castrated males and intact males and females, at doses of 0.1 and 0.2 mg/kg. This difference existed for both tests, although response strength was higher for Test II. Mean alarm call rate for castrated males at the 0.1 mg/kg drug dose in Test I was 33/min, which rose to 62.7 calls/min in Test II. Test II resulted in similarly elevated calling rates over Test I rates at all drug doses tested. These tests also establish that castrated males have a lower threshold drug dose for alarm calls, as neither the 0.1 nor 0.2 mg/kg doses of benactyzine resulted in calling rates significantly elevated above control levels for intact individuals of both sexes.



A regression analysis of 4 intact 'Gothic arch' males indicated the absence of a significant regression trend. However, there was a considerable spread of calling rates among the 4 subjects at all doses.



A regression analysis performed on the 4 castrated males indicated a significant positive correlation between drug dose and calling rate.

D. Hormonal Correlates of Social Behavior in Squirrel Monkeys

This past year, we have begun to analyze the possible correlation between levels of endogenous steroid hormones and behavioral responsivity. Two behavioral paradigms were used. In the first, observations of undisturbed squirrel monkey males housed in groups of 2-3 were made, and vocalizations recorded for subsequent acoustic analysis. A total of 17 adult males housed as 7 groups were studied. Four males housed as a group had been castrated more than 2 years prior to this study. The other subjects were all intact. Groups consisted of either Gothic-arch or Roman-arch squirrel monkey subtypes. Three periods of observation were made. In the first, all subjects were housed in a single room. A group of 8 adult females were also housed in the same room. Vocal recordings were made with the observer sitting in front of a group. All vocalizations were recorded for 45 minutes. Wherever possible, identification of the vocalizer was made on the same tape as the calls themselves, by softly speaking into a hand-held microphone. This observation period was ended when all groups had been observed for at least one 45-minute session. A blood sample was taken from each male at the end of this observation period and prepared for assaying testosterone levels. A second observation period was initiated 3 months later. The purpose of this part of the study was to test for possible seasonal changes in vocal behavior. Some of the subjects used in study 1 were observed under the same conditions, with the females still present in the same room. In addition, 2 Gothic and

2 Roman males were moved to another room, and allowed to live socially with females of their own subtype. Recording sessions again lasted 45 minutes/group. A second blood sample was taken at the end of this observation period. A third study was begun 3 months later. In this case, male vocalizations were recorded while the males were housed apart from any females. A final blood sample was taken at the end of this study. It was predicted that the distribution of call types made by males would differ from those of females under similar social conditions, and that testosterone level would also influence the range and specific types of vocalizations made. However, the latter hypothesis was not substantiated. No significant correlation was found between testosterone level and total vocalizations for any intact male. However, a significant difference was found in vocal behavior between intact males and the 4 castrated males in the study ($X^2_{(5)}=55.840$, $p<.0001$). Castrates produced more chucks and twitters, while intact males produced more err chucks and peeps. The former calls are associated with friendly interactions between established social groups, while the latter calls are associated with excitement and solicitation of contact, particularly between males and females. A comparison of the male castrates with female vocal behavior recorded under similar conditions also indicated significant differences between them. Thus, while the vocal behavior of castrated males is different from that of intact males, and suggestive of some degree of "feminization," it is also not identical to that of intact females.

In the second approach to studying the hormonal correlates of vocal behavior, adult males and females were individually tested for their vocal response to the "defensive threat" paradigm (described in section C, above). Blood samples were taken at approximately 3-month intervals on non-test days and analyzed for testosterone. A single blood draw also was made for cortisol levels. Blood draws were made after administering a small dose of ketamine, and sampling was complete within 5 minutes after initial capture. Mean testosterone levels varied from 5.4 to 30.5 ng/ml of plasma over the course of 1 year. Blood samples from 4 castrated males were analyzed at the same intervals, and showed the expected low levels of testosterone (mean values varying from 0.4 to 1.3 ng/ml). Mean cortisol values were 394 ug/dl for adult females, 244.5 ug/dl for intact adult males and 239.2 for castrated adult males. As reported above, castrated males had significantly higher alarm calling rates than intact males. Examination of the relationship between alarm calling behavior and testosterone values in the intact males did not yield a significant correlation. A test for the relationship between cortisol levels and alarm calling behavior was also performed. A significant positive correlation was found for females but not for males. Since Roman-arch and Gothic-arch females had different mean cortisol levels, a separate analysis was performed on alarm call rate vs. cortisol level within each subspecies. In this case, no significant correlation could be determined. Thus, a correlation between adrenocortical activation and the strength of the vocal response to the alarm test is tenuous at best.

E. Brain Imaging Studies in Marmosets and Squirrel Monkeys

Studies of brain development, a new initiative in the Section begun in FY94, require an actively breeding colony to provide the material for this work. Since we are also conducting studies of behavioral development (Project Z01 HD 01124), the material for associated brain developmental studies is already at hand. Of particular value for correlating behavioral and brain development is the fact that behavioral information is available on the same subjects that enter into the terminal studies associated with brain anatomy. In the future, when a functional imaging

workstation is available in the laboratory, brain development can be studied at several developmental milestones in the same individual. For the present, however, the subjects must be euthanized and their brains prepared for subsequent examination. Some material for these studies comes from infants rejected by their caregivers. However, the best science comes from targeting individuals of specific age, sex and individual experience categories. Animal study protocols designed to provide suitable material for these studies have been approved by the NICHD ACUC.. In FY95, 6 subjects aged 6 months to adult were taken for this work, along with several infants less than 6 months of age.

A major effort was made during FY95 to provide the instrumentation necessary to conduct neuroanatomical research. The departure of Dr. Thomas Insel, an NIMH investigator, last year has temporarily suspended the collaborative studies on the distribution of neuropeptide-containing neurons and their associated brain receptors. However, equipment to continue this work was obtained from the NIMH and is now operating in our laboratory, including a cryostat/microtome for sectioning brain tissue, a high-resolution microscope, and a CCD video camera and computer board for digitizing brain sections and storing them in a computer. Associated software in the computer permits quantitative analysis of radiographically labelled sites in this material, as well as other quantitative studie aimed at determined anatomical differences associated with maturation, gender, experience, and species-specific social organization. Studies directed at this last question are possible due to the availability of material from both marmosets and squirrel monkeys, species which differ in both social organization and involvement of nonmaternal family members in providing infant care.

F. Hormonal correlates of social behavior in marmosets

Twenty-one postpubescent marmosets were included in a study of relationships between the social environment, endogenous hormone levels, and behavior. As reported previously, 12 animals (6 males, 6 females) were removed from their home cages and exposed to an unfamiliar opposite-sex conspecific for 20 minutes in a neutral arena. The pair then remained housed together for 24 hours. A 20-minute observation of social behavior was collected from each animal one day prior to the test, during the neutral arena test, and one day following the test. Femoral blood samples were collected each day; samples were collected 30 minutes and 90 minutes following the neutral arena test. The results indicated that changing social status produced measurable changes in hormone levels, some of which correlated with observable changes in behavior. For example, cortisol levels in these pairs were significantly higher one day after pairing (preseparation mean 230 ug/dl; 1 day post-separation mean 300 ug/dl, p=.005). In the same design, 9 animals (4 males, 5 females) were removed from their home cages, but placed alone into the neutral arena for 20 minutes to "control" for any hormonal and behavioral responses due to the separation from familiar group members, then returned to their home cages after the test. Cortisol sampled from the control animals did not show significant differences (pre-separation mean 173 ug/dl; 1 day post-separation mean 189 ug/dl), suggesting that the increased cortisol of the paired animals was related to the change in social status. Both groups produced high levels of isolation vocalizations during the neutral arena test. However, control animals produced three times more calls while alone in the neutral arena than animals did in the presence of the unfamiliar animal (mean value for paired, 6.5; mean value for control, 26.1). Vocalizations by paired animals remained higher one day after pairing (z=-2.156, p=.031). Further study is planned to examine the effect of a 24-hour separation, without pairing, on vocal behavior and hormones.

The robustness of a physiological response may vary with the strength of pre-existing affiliative bonds. A relationship between affiliative bonds in the social environment and hormones in the marmoset was suggested by changes in cortisol levels of animals removed from family groups, compared to those which were removed from peer groups, after the neutral arena test. The preseparation cortisol values of the two groups (family-housed vs peer-housed) were not significantly different. However, the cortisol response of the animals paired after removal from family groups (n=5) was much greater (preseparation mean 193 ug/dl; 1 day post-separation mean 351 ug/dl) than the cortisol response from animals paired after removal from peer groups (n=7, preseparation mean 238 ug/dl; 1 day post-separation mean 279 ug/dl), while the changes in cortisol from control animals were not significant (family-housed, pre-sep mean, 147ug/dl, post-sep mean 178 ug/dl; peer-housed, pre-sep, 205 ug/dl, post-sep, 202 ug/dl).

The increased testosterone of males found 30 minutes after exposure to unfamiliar females was more pronounced in peer-housed males than family-housed males; overall, testosterone values of the control males did not significantly vary. However, testosterone of the peer-housed males (n=2) rose from a mean of 15 ng/ml to 25 ng/ml and remained high for 24 hours after the neutral arena test, whereas the testosterone of family-housed males was suppressed after their return to the family group (pre-separation mean 22.5 ng/.ml, post-test means 13-15 ng/ml). These results suggest that a physiological response to brief removal from familiar animals may reflect the social organization of the group, and the reproductive "readiness" of each individual in that group.

The effect of social housing was further evidenced by differential isolation calling behavior of family- vs peer-housed animals. Family-housed control animals called more than family-housed paired marmosets in the neutral arena (U=4.5, p=.05), but peer-housed control animals did not call more than peer-housed paired marmosets.

Qualitative differences in bonding between group members may manifest in differing physiological responses to social perturbations. Further study examining the behavioral and physiological responses of breeding, pairbonded individuals to separation is planned. Behavioral and physiological indicators of differential bonding would help indicate appropriate social environments for studying neural mechanisms of social bonding in the marmoset.

Significance to Biomedical Research and the Program of the Institute:

This project has identified brain structures and related neurochemical mechanisms implicated in regulating vocal expression in the contexts of social separation and alarm. The present studies aim at improving our understanding of mechanisms related to the vocal expression of affect in humans, an important but poorly understood aspect of human behavior. Since the principal behavioral probe used in these studies, the primate isolation call, is structurally and functionally homologous to the cry of the human infant, the present investigations provide a comparative approach to studying the infant cry as a marker of normal neurobehavioral development.

Proposed Course:

1) Hormone assays in marmosets and squirrel monkeys. Initially, we will use the ELISA assay for LH that J. Norcross has developed for common marmosets to assess LH levels in adult squirrel monkeys. Once we have demonstrated that this assay is also valid in the squirrel monkey, blood and urine samples will be collected to determine LH cyclicity in adult males and females. We also intent to set up ELISA and RIA assays for gonadal steroids, which we will apply to assessing the levels of these hormones in adult marmosets and squirrel monkeys.

2) Studies of care-giving in marmosets. Based on the data from the juvenile retrieval studies, there appears to be a clear activation of a specific behavior within a proscribed time period. We plan to pursue this study by utilizing c-Fos gene activation techniques to determine particular areas of the brain that may be involved in the mediation of the retrieval behaviors in these young animals.

We plan to perfect the radioimmunoassay techniques and validate the assays for oxytocin, prolactin and vasopressin in marmosets, such that the analysis can be performed on the blood samples we have already collected and correlations can be made with the relevant behavioral data.

Our plans also include the use of intracerebroventricular microinjections of oxytocin, vasopressin, and prolactin to determine the effect of these neuropeptides on specific behaviors, such as infant retrieval. Our efforts to localize the receptors of interest within the brain should facilitate our ICV studies

3) Role of gonadal steroids in mediating the gender differences in cholinergic activation of defensive threat behavior in squirrel monkeys.

We plan to treat 4 castrated males with testosterone, then assess the treatment effects on vocal response to a standardized test for eliciting "alarm" behavior. Other studies will also attempt to temporarily block endogenous aromatase activity in intact males and females, since estrogen is the centrally active steroid hormone in both males and females and is synthesized in the brain by aromatase activity.

Publications:

Moody KM, Steinman JL, Komisaruk BR, Adler NT. Pelvic neurectomy blocks oxytocin-facilitated sexual receptivity in rats. Physiology & Behavior 1994;56:1057-60.

Moody KM, Adler, NT. The role of the uterus and cervix in systemic oxytocin-PGE$_2$ facilitated lordosis behavior. Hormones and Behavior, in press.

Newman JD. Vocal ontogeny in macaques and marmosets: convergent and divergent lines of development. In: Zimmerman E, Newman JD, JŸrgens U, eds. Current Topics in Primate Vocal Communication. New York: Plenum, 1995;73-97.

Newman JD, Farley MJ. An ethologically based, stimulus and gender-sensitive nonhuman primate model for anxiety. Prog. Neuro-psychopharm. Biol. Psychiatry, 1995;17:.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01124-05 LCE

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic and Experiential Influences on the Development of Primate Vocal Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: John D. Newman Section Head, LCE:NICHD

Others:	Moody, Kathleen	IRTA Fellow	LCE:NICHD
	Norcross, Janet	IRTA Fellow	LCE:NICHD
	Smith, Najwa	IRTA Predoctoral Fellow	LCE:NICHD
	Bernhards, Deborah	Bio. Lab. Tech	LCE:NICHD

COOPERATING UNITS *(if any)*

none

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Developmental Neuroethology Section

INSTITUTE AND LOCATION
NICHD, NIH, Poolesville, Maryland 20837

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is currently focussing on gender differences in vocal communication in adulthood and during development, and on the role of individual experience in the acquisition of adult vocal skills. Major findings this year were: 1. Calling rate of infant common marmosets in the context of social separation shows a systematic decline from birth to 3 months, then stabilizes over subsequent monthly assessments. Comparison of vocal behavior in age-matched infants tested in their home colony room and separated from close-range auditory input from the colony indicates that neonates calls less, and older (4-6 month) infants call more, when they are in close audible contact with family members and other familiar conspecifics. 2. The vocal behavior of adult male squirrel monkeys living in same-sex social groups was quantitatively assessed for the first time. Males engage in 'conversational communication,' previously considered to be an exclusively female attribute. However, the incidence of conversational communication in adult males in less than it is in adult females. We currently interpret this gender difference as attributable, at least in part, to early developmental differences in behavioral predispositions between male and female infants.

Project Description:

Objectives:

The principal objective of this project is to understand the roles of genetic mechanisms and individual experience in the development of normal vocal behavior in primates. Current studies are aimed at identifying developmental stages associated with structural maturation of the isolation call, the emergence and differentiation of vocal categories during development, identification of gender differences in vocal behavior, and the structural and functional relationship between the isolation call and other vocalizations in adult and subadult primates.

Methods Employed:

A comparative approach is used in this project, through the study of a variety of nonhuman primate species as well as human infants. Studies of vocal development involve family or peer group recordings, as well as gently separating the infant from its family and placing it in a recording chamber that provides complete visual separation and partial auditory separation from the rest of the colony. Analysis of vocal data consists first of producing time-histories of the tape-recorded vocalizations with a real-time FFT sound spectrograph, then displaying the analyzed vocalizations on a color video monitor or as hard-copy records for later inspection. Temporal and frequency measures of individual vocal utterances or vocal sequences are made either directly off the video monitor, or from the hard-copy recordings using a digitizing tablet and microcomputer. In addition to conventional univariate statistical procedures, linear discriminant function analysis is used for characterizing the acoustic parameters diagnostic for a particular individual, age group, species, population, or behavioral context.

Results

A. Vocal Development in Common Marmosets

The continuing success of breeding in the marmoset colony has provided a robust data base from which questions related to behavioral development in this species can be addressed. Of considerable interest is the emergence of adult vocalizations in the developing marmoset, and the extent to which infantile vocalizations track other measures of development. A catalogue of 8 acoustically distinct types of vocalization have been identified as occurring in neonates during brief periods of social separation from their natal group. A ninth category consists of combinations of 2 or more of the other 8 call types. This last category is principally a characteristic of the first month of life.

A standardized procedure for studying vocal development in captive nonhuman primates developed in this laboratory was applied to 12 (6 male, 6 female) common marmosets. Vocalizations were recorded at birth, 2 weeks, and monthly from 1 to 6 months of age. The testing procedure involved separating an individual from its family group and placing it in a test cage in a room with no other conspecifics for up to 10 minutes. After this brief period of testing, the subject was returned to its family group. During most sessions, subjects vocalized repeatedly, permitting a comparison of calling rates and the distribution of call types produced in this context over an

extended period of development. Calling rates were highest for the day 1 postpartum recording, with a mean of 45.3/min. Calling rate dropped thereafter, averaging 22.1/min at 1 month, 18/min at 2 months, 12.2/min at 3 months, then stabilizing at around 6 calls/min on average at 4, 5 and 6 months of age. Comparison of these values with calling rates of a different group of 6 infants recorded in the colony room (but visually isolated from their family group), reported last year, suggests that the calling rate of separated infants is modulated by the auditory environment. The colony room sessions yielded calling rates of 28.3/min in the 1 day postpartum sample and fairly stable rates of 7.5-9.8/min for the 4, 5 and 6 month age samples. Thus, neonates call less, and older (4-6 month old) infants call more, when they are in close auditory contact with other conspecifics (including their family members), than correspondingly aged infants not in close auditory contact with conspecifics. The modulation of calling rate by the vocalizations of conspecifics would be adaptive in the wild, where neonates are highly dependent on their parents for protection and food, whereas 4-6 month old individuals are weaned and quite agile. In the older group, persistent loud calling would attract potential predators, so a better strategy would be to call at a relatively low rate until other conspecifics are perceived to be close at hand.

In a second study of vocal development in common marmosets, the maturation of context- specificity and gender-specificity in the structure of the phee call was examined. Previous work has shown that the common marmoset isolation call (the phee call) contains both contextual- and gender-specific parameters. Potentially, expression of these characterisics may be related to puberty, but, like some other social behaviors, remain suppressed until the marmosets are in the appropriate social environment. To address the influence of the social environment and the temporal development of these characteristics, phee calls were recorded from 18 postpubescent marmosets (10 females, 8 males) in varying social contexts. Vocalizations were recorded over a period of approximately 5 months. After 1 month, twelve of the animals ("experimentals") were removed from their natal groups and paired with opposite-sex conspecifics; six animals ("controls") remained in their natal groups for the duration of the study. Attempts were made to record from all individuals while they were housed in their natal groups. However, the experimental group produced too few calls during the observation period for statistical analysis. The analysis of home cage calls was limited to calls produced approximately three weeks (Home+3) and thirty weeks (Home+30) after the experimental animals were paired. In addition to home cage recordings, animals were briefly separated for isolation call recordings from their groups three weeks after the study began (Iso-3), and three weeks (Iso+3) and thirty weeks (Iso+30) after pairing. Acoustic parameters of the calls were analyzed by discriminant function analysis for appropriate classification into the known groupings by social status and time into the study. Analysis of calls recorded in each condition showed that 93% were correctly classified by the social status of the vocalizer (range 80-100%). Context differences were also revealed: 92% of the home cage calls and 94% of the isolation calls were correctly classified as to the context in which they were produced. Gender is also apparently encoded in isolation calls recorded from animals separated from their natal groups, as well as after separation from a pairmate. Analysis of calls produced by the experimental animals during isolation indicated that 100% of the calls produced in all three isolation tests were correctly classified by sex. As indicated by cross-validation results, "femaleness" was more discriminable, with ranges of 81-89% correct, than "maleness", which ranged from a low of 44% (Iso+30) to a high of 79% (Iso-3). Home cage call syllables were also correctly classified by sex in 100% of the cases; cross-validation results accurately classed 73% (Home 1) and 82% (Home 2) of the female calls, and 81% and 87% of the male calls, respectively. When tested across all conditions, approximately 95% (range 90-98%) of the syllables produced by experimental animals were correctly

classified. Cross-validation summaries indicated that, when errors did occur, they were most likely to occur within a context. Calls by both sexes were generally distinguishable between contexts (female calls, range 92-100%; male calls, range 92-100%). Cross-validation results indicated that calls recorded from males shortly after pairing (Iso+3) were the least discriminable, being misclassified as coming from an animal vocalizing in its home cage at approximately chance levels. Calls produced by females several months after pairing (Home+30) were the least discriminable female calls, being classified most frequently as Home+3 calls. Limiting analysis to syllables produced by females that became pregnant (4 of 6), 91-100% of their syllables were correctly classifed into conditions. Cross-validation results indicated that calls produced during pregnancy were least discriminable, being classed almost equally across the recording conditions. An analysis of calls produced by females at times of high progesterone ("cycling") compared to calls produced by the same females during low progesterone ("suppressed") indicated that approximately 98% of the calls were correctly classified. Cross-validation results correctly classified 76% of the calls from cycling females and 68% of the calls from noncycling females. These results suggest that both social and hormonal factors influence the structure of the phee call, potentially changing its communicative function.

B. Infant Caregiving in Common Marmosets

1) Juvenile retrieval behaviors: We have determined an earlier onset of juvenile retrieval behavior than previous studies reported. Twenty-five animals were tested at time points ranging from just prior to the birth of their siblings through 60 hours post-partum. As early as 36 hours after their siblings' birth, inexperienced juveniles will retrieve a separated infant in the retrieval paradigm. The latency to retrieve at this point is significantly reduced compared to similar studies prior to a siblings' birth and at 12 hours postpartum (mean 85 sec vs. mean 180 sec and mean 172 sec, respectively). At 60 hours postpartum, the latency to retrieve drops to a mean of 55 sec, which is significantly reduced as compared to the mean of 85 sec at 36 hours postpartum.

2) Family studies: Family groups continue to be tested in the retrieval paradigm. In one study, 8 adult males, 8 adult females, 3 subadults and 2 juveniles were tested with their own infants for retrieval. Immediately after a successful retrieval, a blood sample was taken from the femoral vein. Preliminary results of the retrieval data support earlier findings that adult male and female common marmosets retrieve separated infants with comparable latencies. There are not yet enough data on the younger animals for valid analysis. With the establishment of our new laboratory, we will soon have the capability of analyzing the blood samples using radioimmunoassay techniques to determine whether plasma levels of oxytocin, vasopressin, and/or prolactin are altered in response to successful retrieval behavior.

3) We have utilized three marmoset families with a particular group composition for inclusion in a study aimed at understanding the putative role of prolactin in caregiving behaviors. Each of the groups consisted of an adult male and a pregnant adult female, one set of subadult twins, and one set of juvenile twins. One of the subadults and one of the juveniles were separated from the remainder of the family group and placed into an adjoining cage. The two cages were placed next to each other, such that visual and olfactory contact was possible but tactile contact was not. The separations occured approximately two to four weeks prior to the birth of the newest siblings. Reunion occured approximately two weeks postpartum. Behavioral analysis of the offspring in both cages was performed at various points throughout the separation and subsequent to reunion. Blood sampling was also performed on all the family members before, during, and after separation.

Retrieval tests were conducted on all the subadult and juveniles in two of the groups after the birth of their siblings, during and after reunion. Preliminary analysis suggests that there were significant differences between animals who were separated and those who remained in their family group with respect to several behaviors. Separated animals had significantly longer latencies to retrieve the infants than the animals who remained in the group (101.43 sec vs. 56.84 sec). After reunion, separated animals had significantly shorter latencies to retrieve than before the reunion, but there were no longer differences between separated and nonseparated animals (40.5 sec vs. 62.34 sec). Moreover, separated animals initially carried less than nonseparated animals just after reunion (377.4 sec vs. 1147.6 sec), yet two weeks later, this trend was reversed and separated animals carried significantly more than nonseparated animals due to the fact that the non-separated animals had significantly reduced their carry time (356.2 vs. 37.1). Age differences were also a factor in carrying and retrieval behaviors. Older, experienced animals initially had reduced latencies as compared to younger animals and carried significantly more than younger animals. These trends were again reversed with time, such that younger animals had significantly more carrying time and shorter latencies to retrieve than older animals. It has been suggested by Dixson et al. (1983) that prolactin is increased in fathers who are actively carrying young infants. We hope to determine whether prolactin plays a similar role in the caregiving behaviors of subadult and juvenile siblings and to determine whether changes in prolactin preceed caregiving behaviors or are altered in response to tactile stimulation from the infants.

C. Gender and species differences in squirrel monkey vocal behavior

A new study was initiated this year to determine whether adult male squirrel monkeys engage in the same kind of rudimentary 'conversations' that prior work has shown to exist for adult females. Subjects consisted of intact adult males housed either in same-sex groups or as pairs housed with several adult females. The same-sex groups were studied both with females housed in the same colony room and with females absent. For comparison, 2 all-female groups were studied for their vocal behavior under similar conditions as the males. Since both "Gothic arch" (Saimiri ustus) and "Roman arch" (Saimiri boliviensis) groups were studied, it was possible to also test for species differences in calling behavior, as well.

Species differences:

A significant difference in the type of vocalizations used in quiet social interactions between "Roman" and "Gothic" groups was found, irrespective of gender. For the female groups, "Gothics" produced more peeps, twitters and vits than did "Romans". For the male groups, "Gothics" produced more twitters and chuck/twitter ("hybrid twitter") calls. For males living with females, a significant difference between the 2 species existed only when the females were absent ($X^2(2)=16.637$, $p=.002$), where "Roman" males made more cackle and peep calls.

Gender differences:

Significant differences existed between males and females in the distribution of call types used under similar social circumstances.

"Conversations:"

One of the more interesting aspects of vocal communication in the squirrel monkey is the presence of a conversational mode of communicating, wherein two individuals engage in rapid exchanges of vocalizations. This "conversational subsystem" has been thought to be engaged in exclusively by adult females and used preferentially between two individuals who have established strong mutual affiliation bonds. Observations of developing individuals indicate that they, too, occasionally give short-latency vocal responses to the calls of adult females, suggesting that proficiency in this form of communicating develops gradually with experience. However, prior indication that males did not participate in conversational communication was based on study of captive groups with only a single adult male. Our current study of male social groups has revealed that males do, in fact, engage in conversational communication, albeit at significantly lower levels than adult females. This suggests that conversational communication is related to maintaining affiliative bonds with members of one's own sex and is not a major form of communication between the sexes. The question of the role of individual experience the acquiring proficiency in conversational communication is a subject for future research. One likelihood, and the basis of our current working hypothesis, is that sometime during the first year of life, male and female infants gradually diverge in the amount of time they spend with their mother and other adult females. Males engage in play more than females do and, as a consequence, spend less time sitting quietly in proximity to females. This imparts an advantage to young females in learning, and eventually participating in, the "rules" associated with conversational communication. That adult males have any ability at all in this form of communication may derive from the fact that infants of both sexes spend most of the time either riding on their mother or in close proximity to her during their first weeks of life. Presumably, early learning processes convey some information about conversational communication (including that between mother and infant) that males, as well as females, can access as they mature.

D. Behavior of Juvenile Common Marmosets.

Prior work in this laboratory has established that subadults approximately 1 year of age have low rates of vocalization and sexual behavior, but high rates of play during the time the individuals remain in their natal group with parents present. This past year the behavior of individuals less than 1 year of age was compared with individuals 15-18 months of age living under similar social conditions. The study included 5 (2 male, 3 female) individuals 8-11 months of age ("prepubescent group") and 5 (2 male, 3 female) individuals 15-18 months of age ("postpubescent group"). All observations were made while the focal subject was in their family group. Forty-four observation sessions were made on each group, totaling 22.5 hours. Prepubescent individuals played significantly more than postpubescent individuals (prepubescent group mean, 34.9 min, postpubescent group mean, 12.2 for social play, $z=-2.835$, $p<0.005$, and 26.2 in prepubescents, 9.2 for solitary play in postpubescents, $z=-3.234$, $p=0.002$). In contast, the postpubescent animals showed significantly higher levels of piloerection and territorial displays ($z=-2.932$, $p=0.011$ and $z=-2.537$, $p=0.003$, respectively). These findings indicate that significant changes in play and territorial behavior occur during development in this species and suggest that the onset of puberty may be marked by a significant re-organization of social behavior and underlying neural systems.

E. Vocal Development in Rhesus Macaques.

Having completed a new vocal classification scheme for rhesus infants during FY94, the scheme was applied to 3 data sets, comprised of vocal recordings from infants born in 1991, 1993 and 1994. In all 3 datasets, vocal recordings were made under the standardized conditions of a brief

period of social separation from their mother or peers. In the 1991 and 1993 samples, recordings were made monthly at 3, 4 and 5 months of age. In 1994, younger infants were added to the study, with recordings being made weekly over the first month of life. Offline analysis was performed using "RTS" (Engineering Design) running on a PC. This program permits visual examination of the time x frequency acoustic character of recorded vocalizations as they are played back in "real" time. The program also permits isolating and storing individual calls for more detailed evaluation. All vocalizations of suitable recording quality were assigned to 1 of 22 mutally exclusive categories, based on detailed acoustic characteristics. A single trained technician scored all taped recordings. A total of 29331 calls were scored for the 1991 subjects (7 nursery-reared males, 9 nursery-reared females, 3 mother-reared males, 3 mother-reared females), 34,160 for the 1993 subjects (4 nursery-reared males, 1 nursery-reared female, 6 mother-reared males, 11 mother-reared females), and 40, 575 for the 1994 subjects (5 nursery-reared males, 11 nursery-reared females, 3 mother-reared males, 5 mother-reared females). This makes a total of 104,066 vocalizations in the data set, undoubtedly the largest database of vocalizations available for this species. Socialization of nursery-reared subjects differed between 1991 (in which all nursery-reared individuals were housed in peer groups of 3 or 4 individuals beginning at 1 month of age) and the 1993/1994 subjects, in which subjects were housed individually but given daily exposure to other nursery-reared individuals for 2 hours.

F. Vocal Development in Squirrel Monkeys.

Incorporation of a large number of breeding-age adults that were formerly part of another research program into the Section's colony has permitted initiation of the examination of vocal development in the squirrel monkey, following procedures already being used for rhesus macaque and common marmoset infants. The importance of these new studies stems from the extensive base of prior studies documenting the details of the vocal repertoire in adults of this species, from the fact that more is known about the neural pathways mediating vocal expression in the squirrel monkey than in any other nonhuman primate, and from the availability of an excellent research facility (Building T-18) specifically designed to study vocal behavior in this species. Furthermore, comparative analysis of the patterns of vocal development in the squirrel monkey with those of the common marmoset should give some insight into similarities and differences in behavioral ontogeny in two New World primates with very different social structure. Developmental patterns shared by these two primates are candidates for developmental processes that may be shared by primate infants in general, including preverbal humans.

Significance to Biomedical Research and the Program of the Institute:

The respective roles of experience and genetic mechanisms in the development of normal communication are poorly understood. The availability of primate models for studying how individual experience and genetic transmission of species-specific communication patterns interact provides a unique opportunity for analyzing the mechanisms underlying the expression of communicative behavior in primates. The clear homologies between vocal behavior in the human infant and in the infants of nonhuman primates makes a comparative approach to studying vocal development in humans particularly appropriate.

Proposed Course:

This project will continue to devote major emphasis to tracking the developmental changes in vocal behavior. We will attempt to determine the general principles as well as the variability in developmental milestones in each primate species under investigation (common marmoset, rhesus macaque, squirrel monkey), as well as the emergence of gender-specific and individual differences. Vocal characteristics will be assessed in genetically related individuals, to evaluate the extent of familial traits. In common marmosets and squirrel monkeys, we intend to determine whether the interest of family members toward newborn infants is influenced by the vocal behavior ("crying") of the infant, using playback techniques. Increased emphasis will also be given to the vocal behavior of developing individuals while in their social group. Mother-infant vocal interactions and the differential opportunities to experience adult vocal behavior in young males and females will be studied. The "conversational subsystem" of squirrel monkeys is of particular interest, in that vocalizations used by adult females more frequently engage this communication subsystem than vocalizations used by adult males. The early differentiation of male and female social behavior, including vocalizations, will be studied to identify differences in the maturation rate of vocal behavior in male and female squirrel monkeys. For comparative purposes, the maturation of other vocal subsystems (particularly, the "alarm" and "separation response" subsystems) will also be tracked.

Publications:

Biben M, Bernhards D. Vocal ontogeny of the squirrel monkey, Saimiri boliviensis peruviensis. In: Zimmermann E, Newman JD, Jurgens U, eds. Current Topics in Primate Vocal Communication. New York: Plenum, 1995;99-120.

Zimmermann E., Newman JD, Jurgens U, eds. Current Topics in Primate Vocal Communication. New York: Plenum, 1995;286.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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Psychophysiological Substrates of Cognitive Processing and Socioemotional Expression from 2 to 36 Months

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD
S. A. Linnemeyer	IRTA Fellow	LCE, NICHD

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